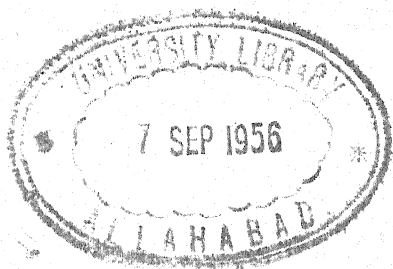


# ANNUAL REVIEW OF BIOCHEMISTRY

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## PREFACE

With the writing of this introduction, the eighth volume of the *Annual Review of Biochemistry* goes to press. We trust that it will receive the same cordial reception with which its predecessors have been greeted.

The usefulness of the *Review* appears to be determined by the extent to which it surveys the progress of investigation in the whole field of biochemical endeavour and also by the extent to which the reviews are critically prepared. Year by year it is becoming increasingly difficult to attain these objectives. Faced by the very evident impossibility of adequately reviewing the greater number of published works in this rapidly expanding discipline, we have felt driven to the alternative solution—the publication of reviews in which, in the judgment of the authors, the contributions of major significance are critically appraised. We have felt encouraged in so doing through the friendly advice of a number of colleagues, in this country and abroad, who have urged upon us the desirability of asking the authors of reviews to adhere to the critical approach and to attempt to give coherent pictures of the present status of the respective subjects.

Such, unquestionably, is the intent of almost all who have been good enough to give so unstintingly of their time in the preparation of these reviews. We are confident that few, indeed, have an inherent preference for the mere cataloguing of papers. We realize, however, that a reviewer, eager to include as much of the published work as space permits, sometimes sacrifices much of the critical quality that is so desirable and, without originally intending to do so, prepares a review which is comprehensive but uncritical.

It is our conviction that the *Annual Review of Physiology*, published as a companion volume, will considerably ease the trials of the reviewer. We hope that in the planning of future volumes of both *Reviews* it will be possible to recognize the deep penetration of biochemistry into physiology by transferring to the *Annual Review of Physiology* much subject matter which, hitherto, the reviewers have felt obliged to include in the *Annual Review of Biochemistry*. The restriction of the latter to investigations of a more chemical character will permit a more precise definition of the field to be covered and will make possible a close integration of the material in both *Reviews*.

To the reviewers, especially, but also to the many who have aided us with advice, by the sending of reprints of their publications to the

authors of the reviews, and by help in many other ways, we extend our warmest thanks. Our sense of gratitude in these matters deepens with the publication of each succeeding volume as we become more and more conscious of the difficulties involved.

We greatly regret that the review on the "Nucleic Acids, Purines, and Pyrimidines," announced in the descriptive circulars, failed to arrive in time for publication.

To Dr. James H. C. Smith we are much indebted for his invaluable help as associate editor.

C. L. A.  
D. R. H.  
J. M. L.  
C. L. A. S.

## ERRATA

Volume V, page 362, formula VII: *insert* N at top of third ring, position 1.

Volume VI, page 533, reference 72: *for* 1576, *read* 1756.

Volume VII, page 39, line 14 from bottom: *for* "Steinhardt has also found that when the effect of temperature on the hydrogen-ion concentration of the solution is taken into account," *read* "Steinhardt has also found that when the effect of temperature on the dissociation-equilibria determining the concentration of a certain most unstable ionic species of the enzyme is taken into account."

Page 49, reference 2: *for* 14, 3 (1937), *read* 14, No. 11, 1 (1937).

Page 203, lines 4 and 5 from bottom: *for* phenyltyrosine, *read* phenylalanine.

Page 246, line 3: *for* Debrue, *read* Delrue.

Page 252, reference 152, *for* Debrue, *read* Delrue.

Page 266, line 14: *for*  $\Delta^5$ -testosterone, *read*  $\Delta^5$ -testosterone.

Page 274, line 20: *for* Westerfield, *read* Westerfeld.

Page 275, line 10: *for* Westerfield, *read* Westerfeld.

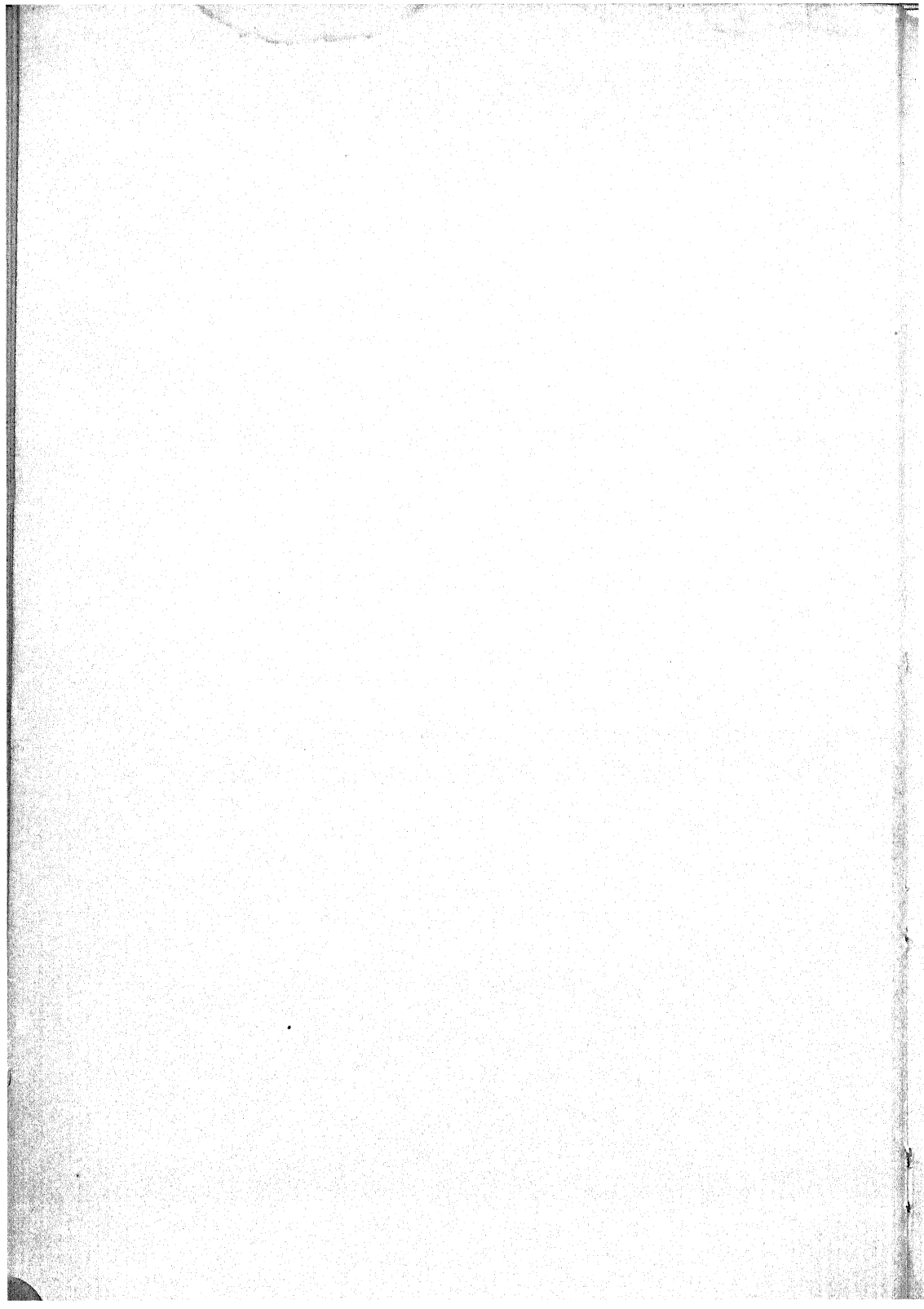
Page 299, reference 155: *for* Westerfield, *read* Westerfeld.

Page 310, line 16: *for* the present formula, *read* the present  $\text{CH}_2$  bridge in the formula.

Page 328, line 10: *delete* and by Quastel and Wheatley (27).

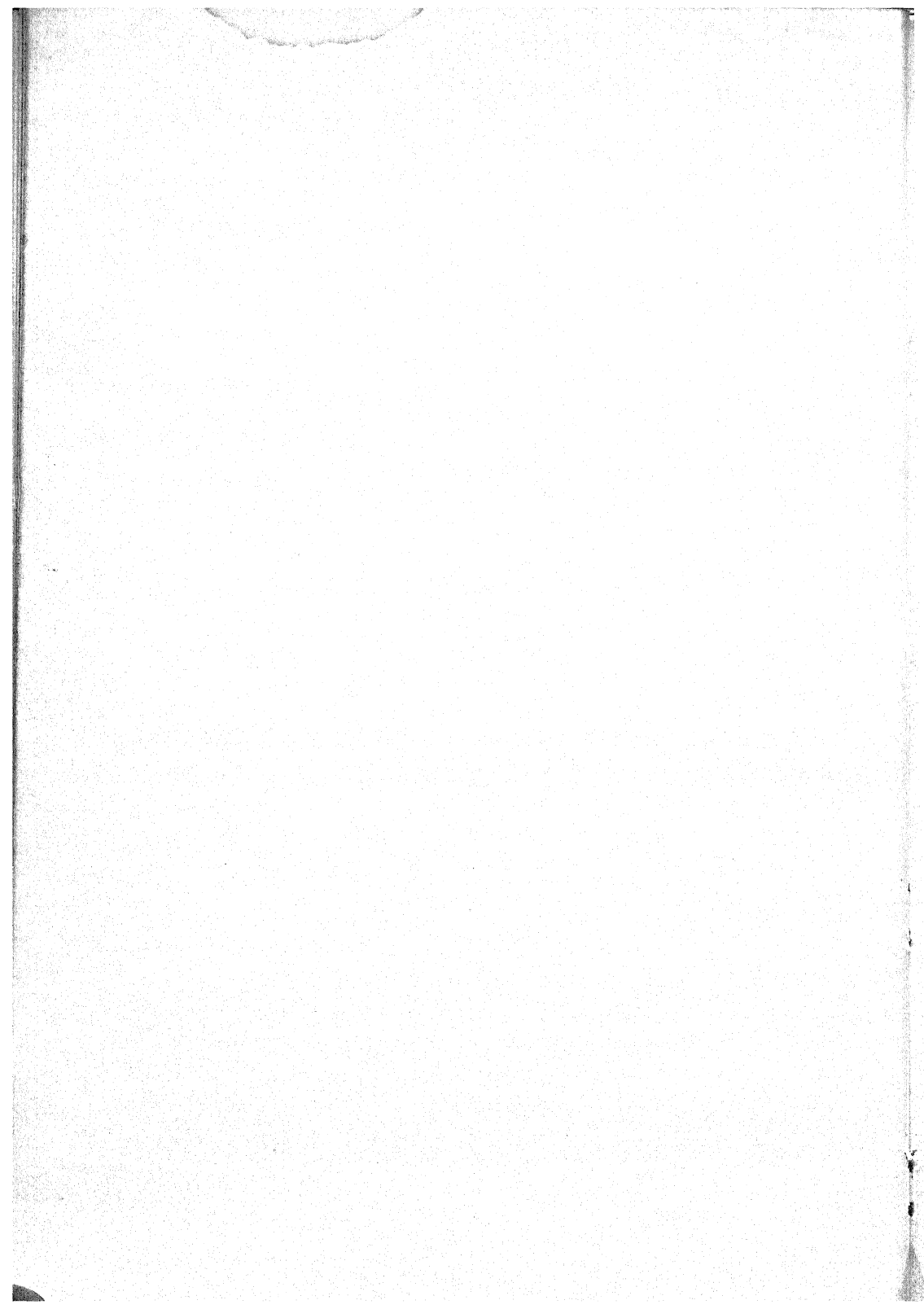
Page 488, reference 3: *for* (1937), *read* (1935).

Page 524, lines 23 and 28: *for* Zimmermann, *read* Zimmerman.



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# BIOLOGICAL OXIDATIONS AND REDUCTIONS

BY MALCOLM DIXON

*Biochemical Laboratory, Cambridge, England*

The rate of progress in this subject during 1938 has been so high that it is only possible in the present article to deal with the more important contributions. Notable advances during the year have been made in the discovery of a new coenzyme nucleotide and of new catalytic flavoproteins, and also in the study of the polyphenol oxidases, the cytochrome system, the mutases and catalase.

## DEHYDROGENASES

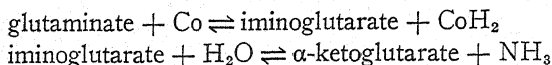
*Alcohol dehydrogenase of yeast.*—This has been isolated as a crystalline protein by Negelein & Wulff (1), who also studied its combination with cozymase, alcohol and aldehyde and the kinetics of the reaction:  $\text{alcohol} + \text{Co} = \text{aldehyde} + \text{CoH}_2$  [Co = cozymase (coenzyme I)]. The dissociation constants (Michaelis constants) for the combination of the enzyme with each of the four reactants are as follows: Co, 0.0001 *M*;  $\text{CoH}_2$ , 0.00003 *M*; aldehyde, 0.0001 *M*; alcohol, 0.024 *M*. These give the concentration of each substance independently at which 50 per cent of the enzyme protein is combined with it. Under normal conditions only a minute fraction of the total cozymase is combined at any instant. At equilibrium, the combined cozymase is half-reduced when the alcohol concentration is 1300 times the aldehyde concentration, though owing to the difference in the affinities for Co and  $\text{CoH}_2$  the free cozymase will be much less than half-reduced. In other words the reduction potential of cozymase must be less negative when combined with the enzyme than in the free state. A similar effect has been found in the case of flavoprotein and free flavin. If the molecular weight of the dehydrogenase protein is assumed to be 70000, one molecule, in presence of excess alcohol and cozymase, will oxidize 17000 molecules of alcohol per minute at 38°.

The properties of the alcohol dehydrogenase of animal tissues, hitherto not much studied, have been investigated by Lutwak-Mann (2) (cf. p. 6). Both she and Quibell (3) find it to be strictly specific for coenzyme I, like the yeast enzyme.

*Glutaminic dehydrogenase.*—This has been studied especially by Euler and his coworkers (4). It oxidizes only the *l*(+)-isomer of glutaminic acid and does not act on other amino acids (5, 6, 7). It is therefore quite distinct from the *d*-amino acid oxidase. The product

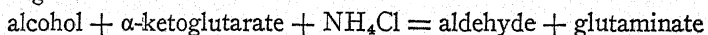


is  $\alpha$ -ketoglutaric acid, which was isolated by Dewan (6) and by Damodaran & Nair (7). The reaction probably takes place as follows:



Co represents the necessary coenzyme. The coenzyme specificity depends on the source of the enzyme: the glutaminic dehydrogenase from yeast (8) and *Esch. coli* (9) work only with coenzyme II, those of plants (5) and animal tissues (5, 6) work only with coenzyme I. In each case the other coenzyme is quite inactive. Euler *et al.* (4) have recently stated, however, in contradiction to their earlier findings (5) and of those of Dewan (6), that the enzyme of liver can work equally well with either coenzyme, and they suggest that possibly liver contains both types of enzyme.

Günther (10) showed by spectroscopic methods that the above reactions are both reversible, for the reduced coenzyme is readily oxidized by a mixture of  $\alpha$ -ketoglutarate and ammonium chloride, though not by either separately. The synthesis of glutaminic acid by this reaction was verified by Adler *et al.* (11), who also demonstrated the reversibility by linking the system with alcohol dehydrogenase through coenzyme I. Thus, in the presence of glutaminic dehydrogenase of liver, alcohol dehydrogenase of yeast and cozymase the following reaction occurred:



The oxidation of alcohol caused the "reductive amination" of the keto acid, cozymase acting as a hydrogen carrier between the two enzymes. Dewan (6) similarly linked the glutaminic and  $\beta$ -hydroxybutyric dehydrogenase systems together through cozymase, and Adler *et al.* (8) linked the glutaminic dehydrogenase of yeast with the hexose-monophosphate dehydrogenase through coenzyme II, for which both of these enzymes are specific.

Euler (4) believes that the glutaminic dehydrogenase is of great biological importance in connection with amino acid synthesis, for when glutaminic acid has been formed from ammonia and  $\alpha$ -ketoglutaric acid other amino acids can then be formed by a direct transfer of its amino group (*Umaminierung*) to keto acids, as shown by Braunstein & Kritzmann (12).

Liver has been found to contain large amounts of glutaminic dehydrogenase, other animal tissues (except kidney) relatively little (6, 4).

*Glycerophosphate dehydrogenase*.—The findings of Green & Dewan (13, 14, 15) that this enzyme from animal tissues requires no coenzyme, and those of Euler *et al.* (16), that it reacts only through coenzyme I, have now been reconciled by Adler *et al.* (17). Muscle contains two  $\alpha$ -glycerophosphate dehydrogenases: a soluble one which is specific for coenzyme I, and an insoluble one (that of Green) which does not react with coenzyme I. The former cannot be detected in muscle extracts unless flavoprotein or diaphorase (cf. p. 11) is added as well as cozymase, while in Green's method of preparation the soluble dehydrogenase is washed away leaving only the insoluble one.

The coenzyme specificity of the dehydrogenases presents an interesting problem. With one or possibly two exceptions, each dehydrogenase can react only with one coenzyme. But several pairs of dehydrogenases occur, in which the members of each pair show identical behaviour towards the substrate but differ sharply in their coenzyme specificity. Several such pairs are shown in Table I. No explanation is at present forthcoming.

TABLE I  
COENZYME SPECIFICITY OF DEHYDROGENASES

Dehydrogenase	Source	Coenzyme
Lactic	Yeast	—
Lactic	Muscle	I
$\alpha$ -glycerophosphate	Muscle (soluble)	I
$\alpha$ -glycerophosphate	Muscle (insoluble)	—
Glutaminic	Yeast, Bacteria	II
Glutaminic	Liver, Plants	I
Glucose	Liver	I or II
Glucose	<i>Aspergillus</i>	—
Succinic	Muscle	—
$\alpha$ -hydroxyglutaric	Animal tissues	—
Malic	Muscle	I
Aldehyde	Milk, Liver	—
Alcohol	Yeast, Liver	I
Triosephosphate	Yeast, Muscle	I
Hexosemonophosphate	Yeast	II
Phosphohexonic	Yeast	II
<i>d</i> -amino acid	Animal tissues	F

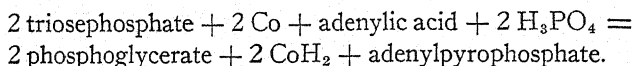
(—) denotes that the enzyme does not work through coenzyme I or II and that there is no evidence that any coenzyme is required.

I denotes that the enzyme works through coenzyme I (cozymase, diphosphopyridine-nucleotide) and is inactive with coenzyme II.

II denotes that the enzyme works through coenzyme II (triphosphopyridine-nucleotide) and is inactive with coenzyme I.

F denotes that the enzyme works through the flavin dinucleotide described below.

*Triosephosphate-dehydrogenase* (cf. also p. 19).—An important special property of this enzyme has been brought to light by Meyerhof (18, 19, 20) and Needham & Pillai (21, 22) independently. Both in muscle and yeast the oxidation of triosephosphate to phosphoglyceric acid by the dehydrogenase in presence of cozymase is coupled with the synthesis of adenylypyrophosphate from inorganic phosphate and adenylic acid. The following reaction was shown to occur stoichiometrically:



Thus the oxidation and the esterification are coupled together by some mechanism not yet understood, so that free energy liberated by the oxidation is used for the synthesis of the adenylypyrophosphate. Meyerhof (20) has shown that the reaction can proceed in the reverse direction, in which case the energy of breakdown of adenylypyrophosphate is used for the resynthesis of carbohydrate from phosphoglycerate through triosephosphate. In muscle, at any rate, the oxidation of triosephosphate can go on to some extent in the absence of adenylic acid and phosphate, but the velocity of oxidation is much greater if the esterification is allowed to proceed at the same time. Arsenate breaks the coupling completely.

The coupling appears to be peculiar to this enzyme—no such effect is shown even by the very closely related triose dehydrogenase (22, 23). It therefore appears to be specifically connected with the oxidation of triosephosphate, rather than with the reduction of cozymase, which is common to both systems. This is of especial interest in view of the central place which the triosephosphate dehydrogenase occupies in carbohydrate breakdown. The effect gives an important clue to the manner of utilization of the energy liberated by oxidations in the living cell.

Adler & Günther (24) have confirmed the effects with the yeast dehydrogenase by spectrophotometric estimations of  $\text{CoH}_2$ , but have shown that the process is not a simple equilibrium reaction, for the final  $\text{CoH}_2$  concentration does not depend on the concentration of triosephosphate or phosphoglycerate. The elucidation of the mechanism will be awaited with interest.

*Phosphohexonic dehydrogenase*.—This enzyme from yeast has been further studied by Dickens (25). Like the hexosemonophosphate dehydrogenase, it acts through coenzyme II, but the two enzymes are

distinct. Evidence supporting Dickens' theory of hexosemonophosphate breakdown has been obtained. Hexosemonophosphate is first oxidized by its dehydrogenase and coenzyme II to phosphohexonic acid. This is then oxidized further by the phosphohexonic enzyme and coenzyme II with the loss of two hydrogen atoms per molecule, giving apparently ketophosphohexonic acid which undergoes decarboxylation to form pentosemonophosphate. The cycle is then repeated with the production of a tetrosemonophosphate and so on. The first stage in this second cycle is due to a pentosemonophosphate dehydrogenase, also found by Dickens in yeast, which works with coenzyme II but appears to be distinct from the hexosemonophosphate enzyme. Dickens finds, however, that the pentosemonophosphate most readily oxidized is not the arabinose phosphate which would be expected to be formed from hexosemonophosphate, but *d*-ribosephosphate. This is readily oxidized and fermented although *d*-ribose itself is unattacked.

Engelhardt & Barchash (26) have also studied the oxidation of phosphohexonic acid.

*Aldehyde (xanthine) dehydrogenase.*—A large amount of evidence that the xanthine and aldehyde oxidases are identical has been brought together by Dixon (27), who concludes that the identity is now finally proved.

Ball (28), working in Warburg's laboratory, states that the xanthine oxidase is a flavoprotein, on the following evidence: Active preparations<sup>1</sup> from milk showed a flavin spectrum and the characteristic photoderivative could be isolated; if hypoxanthine were added to the enzyme anaerobically the flavoprotein was reduced, but was reoxidized on admitting oxygen; it was not possible to separate the protein and flavin components and to regenerate the enzyme by recombining them, but a preparation was obtained which showed a three- to four-fold increase of oxygen uptake on adding a fraction containing flavin, though it was found that both the purified flavin nucleotides were inactive.

So far only this preliminary note has appeared, and without the full evidence it cannot be taken as conclusive. The mere presence of a flavoprotein and the reduction by hypoxanthine do not show that the flavoprotein is identical with the enzyme, since it might be acting simply as a hydrogen acceptor. The increased uptake on mixing the two fractions might also be explained on other lines, e.g., by protec-

<sup>1</sup> The activity was the same as that reached by Dixon & Kodama (29) in 1926.

tion of the enzyme against the hydrogen peroxide formed in the reaction. Warburg (30) has since stated that the oxidase could not yet be resynthesized from its protein and flavin "components."

Ball's suggestion is however supported by Corran & Green (31), who have obtained a flavoprotein (about 50 per cent pure) from similar xanthine-oxidase preparations (see p. 14). They stated at first that it possessed no xanthine oxidase activity, but now find their preparations to be active and believe that it may be the oxidase. They find however (contrary to Ball) that it is not reduced anaerobically by hypoxanthine. The evidence is thus somewhat contradictory.

The question is one of some theoretical importance, since this enzyme is one of the few dehydrogenases which appear to react with oxygen without coenzymes or carriers; but until further evidence appears it must be left undecided. Booth (32) has carried out further studies on the specificity of the enzyme towards aldehydes and purines.

*Choline dehydrogenase of liver.*—This has been studied by Mann *et al.* (33) and shown to be a typical dehydrogenase of the succinic dehydrogenase class. It reacts with oxygen through the cytochrome system and reduces cytochrome-*c* directly, apparently without the need for any coenzyme. The oxygen uptake is inhibited by cyanide, owing to poisoning of the cytochrome oxidase, but the dehydrogenase itself is unaffected. It also oxidizes arsenocholine.

*Specific inhibitors, (a) Iodoacetic acid.*—This acts in strong solution as a general enzyme poison, but in dilute solution ( $< 0.001 M$ ) its action is remarkably specific. Its effect on dehydrogenases has been studied by Dixon (34) who found that the alcohol dehydrogenase of yeast was sharply differentiated from the others by its high sensitivity to iodoacetate. No other dehydrogenase tested was affected by dilute solutions, with the exception of the triosephosphate dehydrogenase of yeast, which was poisoned to some extent. Shortly afterwards Green *et al.* (35) found that the triosephosphate dehydrogenase of muscle showed a very high sensitivity to iodoacetate. These facts explain the effect of the latter on glycolysis and fermentation, which are inhibited at the stage known to be catalysed by these enzymes. Dixon (34) showed that the iodoacetate acted on the enzyme itself, and not on the coenzyme or any enzyme-coenzyme complex.

The above facts were confirmed by Euler *et al.* (36). Lutwak-Mann (2) finds however that the alcohol dehydrogenase of animal tissues is sharply differentiated from that of yeast in being highly resistant to iodoacetate even in  $0.01 M$  solution.

Iodoacetate was also shown by Dixon & Lutwak-Mann (37) to poison aldehyde mutase (see p. 17).

*Specific inhibitors, (b) Glutathione; sulphydryl groups in dehydrogenases.*—Hopkins and his coworkers (38, 39) have made the interesting observation that incubating the succinic dehydrogenase with high concentrations of oxidized glutathione (GSSG) renders it completely inactive, but that it regains its original activity on treatment with reduced glutathione (GSH). They obtained no trace of such an effect with any other dehydrogenase or with aldehyde mutase or cytochrome oxidase. These results suggest that succinic dehydrogenase contains sulphydryl groups;<sup>2</sup> it becomes inactive when these are oxidized by GSSG, and becomes once more active when they are reduced by GSH. The effects were not due to traces of copper in the glutathione. Treatment with alloxan, which is known to oxidize sulphydryl groups, also inactivates the enzyme, and again it is reactivated by treatment with GSH. Dibasic acids (malonic, fumaric, succinic itself and pyrophosphoric) were already known from competitive inhibition effects to combine with the enzyme, and it was found that they protected it against inactivation by GSSG. In the case of malonate, at any rate, even a concentration of 0.0002 *M* gave a high degree of protection. In all these experiments the substances used to oxidize or reduce the enzyme were washed away before the activity was tested.

Morgan & Friedmann (41) found that maleic acid combines with sulphydryl groups, and previous treatment with maleate (but not fumarate) also inhibits the enzyme.

Euler & Hellström (42, 43) obtained somewhat similar though less marked effects by oxidizing the enzyme with ferricyanide and reducing it with hyposulphite. It is not clear however whether these were due to a real action on the enzyme, for the reagents were not removed before the activity tests were done, and their presence might have affected the velocity. Euler suggests that the sulphur groups of the enzyme act by being alternately reduced by succinate and reoxidized by cytochrome, thus catalysing the transport of hydrogen. This however ignores the fact that once the sulphydryl groups have been oxidized (*e.g.*, by GSSG) the enzyme is totally inactive towards succinate, and it only becomes active when they are once more reduced

<sup>2</sup> Hellström (40) had suggested shortly before that the activity of this enzyme was due to dithio groups which were reduced by succinate, but produced no evidence to support the suggestion.

by GSH. Were Euler's view correct, succinate should reduce them after GSSG treatment and no inactivation should be observed. It is clear that the sulphur groups do not undergo a  $-\text{SH} \rightleftharpoons -\text{SS}-$  cycle during the catalytic action of the enzyme.

Rapkin (45) has recently shown that the triosephosphate dehydrogenase of muscle behaves in much the same way as the succinic enzyme, being partially inactivated by incubation with GSSG and reactivated by treatment with cysteine and to a less extent by GSH. It is also inactivated by iodine, and under certain conditions can then be reactivated by hydrogen sulphide. Rapkin concludes that sulphydryl groups are involved in this enzyme also.

Iodoacetate reacts with sulphydryl groups, and it might be expected that there would be a general parallelism between the inactivation of dehydrogenases by iodoacetate and GSSG. It does not however appear that this is the case. It is true that triosephosphate dehydrogenase is highly sensitive to iodoacetate. Succinic dehydrogenase is quite insensitive, however, to small concentrations, and Hopkins found that even after incubating in 0.04 *M* iodoacetate for one hour more than 10 per cent of its activity remained. Under these conditions many other enzymes are destroyed. Dixon (34) found that both glycerophosphate and lactic dehydrogenases are more sensitive to high concentrations of iodoacetate than the succinic enzyme, although neither is inhibited by GSSG. The aldehyde mutase also, which is quite insensitive to iodoacetate, is altogether resistant to GSSG. The highly sensitive alcohol dehydrogenase of yeast does not appear to have been tested with GSSG.

Iodoacetate reacts rather slowly with sulphydryl groups combined in proteins and it is very possible that its specific action on the alcohol and triosephosphate dehydrogenases is due to some other mechanism.

*Specific inhibitors, (c) Quinoneimine.*—Bernheim *et al.* (46, 47) found that quinoneimine ( $\text{O}:\text{C}_6\text{H}_4:\text{NH}$ ), the oxidation product of *p*-aminophenol, specifically inhibits the xanthine oxidase (dehydrogenase) of liver, even in very small concentration. A concentration of 0.00001 *M* produced a 50 per cent inhibition, and with higher concentrations the inhibition was complete. The other common dehydrogenases were not affected, and closely related compounds had no effect on the xanthine oxidase, so that the reaction is very specific. Milk contains a substance which interferes, so that the results with the milk oxidase were variable. Uricase was unaffected, guanase slightly inhibited.

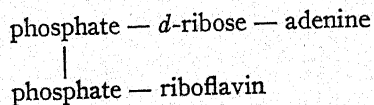


Combining the above results on specific dehydrogenase inhibitors with previous work (48) we may sum up as follows: cyanide, purines and quinoneimine inhibit specifically the xanthine dehydrogenase; pyrophosphate inhibits the succinic dehydrogenase; GSSG inhibits the succinic and triosephosphate dehydrogenases; and iodoacetate inhibits the triosephosphate and yeast alcohol dehydrogenases.

*Technique.*—Quastel & Wheatley (44) have introduced a useful manometric method of studying anaërobic oxidations by dehydrogenases, using ferricyanide as hydrogen acceptor in presence of bicarbonate. Ferricyanide is readily reduced to ferrocyanide by dehydrogenase systems, and as one molecule of acid is liberated in the process it can be followed manometrically by the liberation of carbon dioxide from the bicarbonate. Ferricyanide is practically non-toxic, and as it is reduced directly by the reduced forms of the coenzymes, and also by those systems which do not involve coenzymes, the reaction is independent of both the cytochrome and flavoprotein systems. Allowance must be made, of course, for any acid oxidation products of the substrate.

#### COENZYMES

*Coenzyme of d-amino acid oxidase.*—Much interest has been created by the isolation and identification of this coenzyme. Das (49) in 1936 had shown the existence of a dialysable coenzyme of the amino acid oxidase and had shown that it was different from the known coenzymes. This was confirmed at the beginning of 1938 by Warburg & Christian (50). Shortly afterwards, Straub (51) described the preparation of the highly concentrated coenzyme and stated that it contained a flavin group and that it was ubiquitous in animal tissues. A few days later Warburg & Christian (52) reported the isolation of a barium salt containing one flavin and two adenine groups, probably in the form of a nucleotide, but showed later (53, 54) that this was impure and that the pure substance was a flavin-adenine-dinucleotide. It was obtained from yeast (55) as well as from animal tissues. Its structure is probably



It may be regarded either as cozymase in which the nicotinic amide is replaced by the alloxazine group, or as a compound of adenylic acid



with lactoflavinphosphate.<sup>3</sup> It is a third dinucleotide acting as codehydrogenase.

A full description of its properties and method of isolation has recently been given by Warburg & Christian (56). Measurements of the affinity of the oxidase for the coenzyme show that half the enzyme is combined with coenzyme when the concentration of free coenzyme is  $2.5 \times 10^{-7} M$ . The affinity is therefore very much greater than that of other dehydrogenases for their coenzymes (coenzymes I and II). On the other hand the complex is more dissociable than the other flavoproteins. In presence of excess of enzyme one molecule of coenzyme transports 1440 molecules of oxygen per minute at 38°.

The obvious hypothesis is that the coenzyme acts by becoming alternately reduced by the amino acid substrate and reoxidized by oxygen, by analogy with other flavin systems. It was found (56), however, that it was not reduced anaerobically by the enzyme and amino acid. Reduction could only be observed if very large amounts of the oxidase were added (amounts stoichiometrically equivalent to the coenzyme). The explanation suggested by Warburg is that, when reduced, the coenzyme remains attached to the enzyme, so that the remainder is kept away and cannot be reduced.

This coenzyme is especially interesting because it also combines with other proteins giving flavoproteins ("yellow enzymes") which act in the same way as the original yeast flavoprotein and much more efficiently. That is to say they catalyse the oxidation of the reduced forms of coenzymes I and II by respiratory carriers. This reaction is not however catalysed by the amino acid oxidase-coenzyme complex (see p. 16).

*Deaminocozymase.*—This is cozymase with the amino group of the adenine removed by nitrous acid treatment, and has been found by Euler *et al.* (58, 59, 60) to act instead of cozymase in fermentation and certain dehydrogenase reactions. Its activity is however less than 40 per cent of that of cozymase itself.

*Conversion of coenzyme I into coenzyme II.*—Some evidence of this has been obtained by Euler *et al.* (61, 62, 63). If cozymase were incubated with adenylypyrophosphate and apozymase, and the protein removed by heat coagulation, the solution contained a substance which acted as coenzyme with the hexosemonophosphate dehydrogenase. As

<sup>3</sup> Karrer *et al.* (57) had suspected the presence of this compound in their lactoflavinphosphate preparations in 1937.

the latter is specific for coenzyme II, the results indicated that a few per cent of the cozymase had been converted into coenzyme II, presumably by phosphorylation at the expense of the adenylypyrophosphate. Indications of the reverse transformation were also obtained, as coenzyme II, after a latent period, could activate fermentation by apozymase, a process which is specifically catalysed by coenzyme I.

*Dephosphorylation of cozymase by phosphatases.*—This has been studied by Schlenk, Günther & Euler (64). More than one type of phosphatase is concerned. They suggest that the first and slowest step is a splitting into two mononucleotides by a pyrophosphatase, followed immediately by a splitting off of inorganic phosphate by a nucleotidase. Most phosphatase preparations contain nucleosidases, but by using a special nucleosidase-free preparation they obtained intact the nicotinic amide nucleoside from cozymase. This was found to have no coenzyme properties.

Improved methods of preparation of cozymase and reduced cozymase are given by Schlenk (65) and Ohlmeyer (66) respectively.

A substance which activates the oxidation of pyruvate by oxygen or methylene blue in presence of enzyme preparations from muscle has been isolated in crystalline form by Annau (67).<sup>4</sup>

#### DIAPHORASE; COENZYME FACTOR

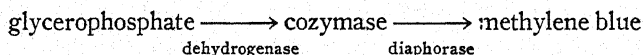
An important link in the respiratory mechanism of animal tissues has recently been brought to light by the discovery of the catalyst called "diaphorase" by Euler and "coenzyme factor" by Green, and this appears to have solved the problem of the way in which the coenzyme-specific dehydrogenases react with oxygen. Reduced coenzymes I and II do not by themselves react either with oxygen, with respiratory carriers like cytochrome, or with hydrogen acceptors like methylene blue. In yeast they can react with the flavoprotein of Warburg & Christian (68), and this can in turn react with oxygen either directly or, more rapidly, through the cytochrome system (69). In animal tissues, however, this flavoprotein has not been found, and Ogston & Green (70) only obtained appreciable reduction of cytochrome-*c* with those dehydrogenases which do not involve coenzymes I or II. The mechanism by which the reduced coenzymes react with oxygen has therefore been obscure hitherto.

Green *et al.* (71, 72) showed that animal tissues contain a cyanide-sensitive "coenzyme oxidase" which catalyses the oxidation of reduced

<sup>4</sup> This has now been identified as succinic acid (164).

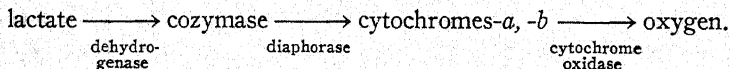
coenzyme I by oxygen. The amounts of flavoprotein present were too small to be detected, although the amounts of yeast flavoprotein required to produce the same activity would have been quite large. A new catalyst was therefore involved.

The nature of the "coenzyme oxidase" system has now been cleared up. Euler *et al.* (73, 74) obtained a preparation from muscle in which they could not detect flavoprotein but which nevertheless catalysed the reduction of methylene blue by reduced cozymase. This catalyst, which they later (75) called "diaphorase," differed from yeast flavoprotein in being unable to catalyse the oxidation of reduced coenzyme by oxygen. It was cyanide-stable. They showed that the soluble  $\alpha$ -glycerophosphate dehydrogenase together with its substrate and cozymase could only reduce methylene blue if diaphorase was also added. The reaction is evidently:



The arrows show the direction of hydrogen transfer. They stated that the diaphorase could also reduce cytochrome-*c*.

Dewan & Green (76, 35, 77), independently and almost simultaneously with Euler, discovered the same catalyst, which they called "coenzyme factor"; they showed that it played an essential part in the reduction of methylene blue by the lactic, malic,  $\beta$ -hydroxybutyric, alcohol, triosephosphate and triose dehydrogenases (all involving cozymase) and could also act with the hexosemonophosphate dehydrogenase (involving coenzyme II). They showed, moreover, that it formed a component of their "coenzyme oxidase," the other component being the cytochrome system. Cytochrome-*c*, however, appears to play no part in the "coenzyme oxidase" system, as its rate of reduction by coenzyme + diaphorase is too low. The active components are cytochromes-*a* and -*b*, which are rapidly reduced by the diaphorase-coenzyme system and reoxidised by the cytochrome oxidase + oxygen. The cytochrome oxidase, and therefore the "coenzyme oxidase," is cyanide-sensitive. Thus, taking the oxidation of lactate as a typical example, the complete system is as follows:



Dewan & Green's results showed clearly that diaphorase was very much more active than yeast flavoprotein, weight for weight.

This is the first time a definite function has been shown for cytochromes-*a* and -*b*.

Diaphorase apparently occurs in all animal tissues (78). Green & Dewan (79), and later Euler & Günther (80), also prepared it, apparently free from flavoprotein, from yeast. It also occurs in bacteria (79).

Euler (78, 80), using spectrophotometric methods, found that diaphorase oxidised only coenzyme I and was unable to act on coenzyme II. Green (76, 77) on the other hand found that it readily catalysed the reduction of methylene blue by the hexosemonophosphate system, which acts only through coenzyme II. The cause of this discrepancy is not clear. Ordinary yeast flavoprotein reacts with both coenzymes.

Dewan & Green (77) suggest that diaphorase is an enzyme which acts on the reduced coenzyme in much the same way as, for instance, the succinic dehydrogenase acts on its substrate, that it is in fact a dehydrogenase of reduced coenzyme.

The nature of diaphorase is now clear. Straub (81) has succeeded in isolating from heart muscle a new flavoprotein, the prosthetic group of which is the flavin-adenine-dinucleotide which also acts as the coenzyme of the *D*-amino acid oxidase. Straub, Corran & Green (82) have now shown that this is identical with diaphorase. Its activity is very high: one molecule of the combined flavin catalyses the oxidation of 8500 molecules of reduced cozymase per minute at 38° under optimum conditions ( $Q_{0_2} = 180000$ ). This high activity explains why diaphorase preparations had previously been obtained which were still active although the amount of flavoprotein was too small to detect. The catalytic efficiency and the concentration in tissues of the new flavoprotein are sufficiently high to permit the conclusion that it plays a fundamental rôle in cellular respiration.

Straub, Corran & Green made the statement that the diaphorase flavoprotein does not undergo a cycle of oxidation and reduction during its catalytic action, but they have recently (165) withdrawn this statement and now find that the rates of oxidation and reduction are sufficient to account for its catalytic action.

The identification of diaphorase as a flavoprotein brings a somewhat greater uniformity into the subject, for it now appears that the oxidation of the reduced coenzymes by cytochrome is always catalysed by one or other of the flavoproteins.

Catalysis by a flavoprotein is always necessary to enable coenzymes

I or II to reduce methylene blue, but this is not always the case with other hydrogen acceptors. Dickens & McIlwain (83) have shown that phenazine dyes react directly with coenzyme in the hexosemonophosphate system and act as carriers in the oxidation, even in the absence of flavoprotein. Kubowitz (84) showed that *o*-quinone acted in the same way with cozymase in the alcohol system. It is perhaps significant that phenazines and quinones share with flavins the somewhat rare property of forming semiquinones; *i.e.*, the reaction, although involving two hydrogen atoms per molecule, can take place in two successive stages each involving one hydrogen atom. As mentioned previously, Quastel (44) showed that ferricyanide oxidizes the coenzyme directly.

### FLAVOPROTEINS ("YELLOW ENZYMES")

Several new flavoproteins have been discovered during the year. In most, if not all, of these the prosthetic group consists of the flavin-adenine-dinucleotide (coenzyme of the amino acid oxidase). Previously only one flavoprotein was known, of which the prosthetic group is lactoflavinphosphate.

Corran & Green (85, 31) have isolated a flavoprotein from concentrated xanthine oxidase preparations obtained from cows' milk by Dixon & Kodama's (29) method. They estimate that 100 litres of milk may contain about 2.5 gm of the flavoprotein. Their purest preparations contained 0.53 per cent of combined lactoflavinphosphate, and ultracentrifugal measurements showed that they contained about 45 per cent of the pure flavoprotein, which must therefore contain about 1.2 per cent of flavinphosphate. This is about twice as much as the original yeast flavoprotein. The milk flavoprotein is orange-red in colour, quite unlike the yellow of other flavoproteins. It is reduced by hyposulphite and reoxidized by oxygen. The molecular weight is 280,000 as determined in the ultracentrifuge by Philpot (see 31). Other properties are given in Table II.

According to Corran & Green the prosthetic group is probably the flavin-adenine-dinucleotide. When split off from the protein it can act as well as the natural coenzyme in the amino acid oxidase system. They state however that the properties of the two nucleotides do not seem to be quite identical.

The milk flavoprotein rapidly catalyses the oxidation of reduced cozymase by methylene blue, like diaphorase and other flavoproteins.



It is more than ten times as efficient as the original yeast flavoprotein as judged by the maximum "turnover number." This number, which probably forms the best comparison of catalytic activities, was originally defined as the number of times the catalyst is oxidized and reduced per minute, but now, for reasons given below, it is better taken as the hydrogen equivalent of the methylene blue reduced per minute divided by the hydrogen equivalent of the flavin itself.

TABLE II  
PROPERTIES OF FLAVOPROTEINS

	<i>a</i> *	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>
Source .....	yeast	milk	yeast	<i>a</i> + <i>c</i>	heart	kidney
Prosthetic groups† ...	M	D?	D	D	D	D
Colour .....	yellow	orange-red	greenish yellow		yellow	
Absorption bands at..	380; 465	350; 450	377; 455	380; 465	359; 451	—
Mol. wt. per flavin group .....	78,000	38,000	60,000			
Mol. wt. ....	78,000	280,000				
Flavin groups per mol.	1	8				
Max. turnover number	50	550	> 50	ca.35	8,500	1,440
Reacts with:						
Methylene blue ....	+	+	+	+	+	+
Oxygen .....	+	+—‡	—	+	—	+
Cytochrome- <i>a</i> , - <i>b</i> ..					+	
Cytochrome- <i>c</i> .....	+	+	+—§		—	
Red. coenzyme I...	+	+			+	—
Red. coenzyme II..	+	+?	+	+	—+¶	—

\* *a* = Flavoprotein of Warburg & Christian (68)

*b* = Flavoprotein of Corran & Green (85)

*c* = Flavoprotein of Haas (86)

*d* = Mixed flavoprotein [protein of *a* + prosthetic group of *c*; Warburg (30)]

*e* = Flavoprotein of Straub (81) (= diaphorase)

*f* = Amino acid oxidase

† M denotes flavinmononucleotide (lactoflavinphosphate); D denotes flavin-adenine-dinucleotide.

‡ Reduced form is oxidized by O<sub>2</sub>, but does not catalyse oxidation of coenzyme by O<sub>2</sub>.

§ — when very pure.

¶ — (Euler), + (Green).

Corran & Green have made some remarkable observations on the mode of action of this flavoprotein, which appear to show that it does not act catalytically by becoming alternately reduced and oxidized. The reduced form is instantaneously oxidized by oxygen, yet it cannot catalyse the reaction of cozymase with oxygen, but only with methy-

lene blue, etc. On the other hand the oxidized form is not reduced by excess of reduced cozymase in the absence of oxygen: reduction did not occur even in one hour, whereas it should have been completely reduced in one second to account for the observed rate of catalysis. Similar observations were made with the original yeast flavoprotein of Warburg & Christian. It is true that this undergoes a cycle of oxidation and reduction, but this is not sufficiently rapid to account for the observed catalysis.

The view of Corran & Green is that the milk flavoprotein remains in the oxidized form throughout the catalysis; the reduced coenzyme combines with it, and when in the combined state can be oxidized directly by methylene blue. In other words the flavoprotein activates the cozymase precisely in the same way as a dehydrogenase activates its substrate. If this view is established it will effect a fundamental change of ideas on the part played by flavoproteins.

Haas (86) has recently isolated a second flavoprotein from yeast, of which both the protein and the prosthetic parts are different from the original yeast flavoprotein. Both portions were obtained separately, and the active flavoprotein was re-formed on bringing them together. The prosthetic group is again the flavin-adenine-dinucleotide; lactoflavinphosphate is inactive with this protein. It catalyses the reaction between reduced coenzyme II and methylene blue, but not oxygen, although its reduced form is oxidized by oxygen (*cf.* milk flavoprotein). It reduces cytochrome-*c*, but loses this property when it is highly purified.

Warburg & Christian (30) have found that the flavin-adenine-dinucleotide unites with the protein of the original yeast flavoprotein (the normal prosthetic group of which is lactoflavinphosphate) to give a new catalytically active flavoprotein. Its properties closely resemble those of the original flavoprotein, in spite of having a different prosthetic group, which shows the decisive influence of the protein in determining the properties. This is still more clearly shown by the fact that the compound of the same prosthetic group with the protein of the amino acid oxidase is totally unable to oxidize coenzymes I and II, while the flavoproteins are unable to oxidize amino acids.

The flavoprotein of animal tissues (diaphorase), very recently isolated by Straub (81), has already been mentioned, and further properties are shown in the table. The flavin content of Straub's preparations is 0.7 per cent, expressed as lactoflavinphosphate.

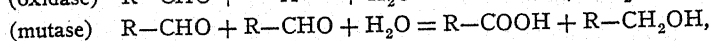
The amino acid oxidase has been included in the table for comparison, though it differs from the other flavoproteins in many respects. The possibility that *b* may be identical with xanthine oxidase has been mentioned above.

Kuhn & Desnuelle (87, 88) have isolated a number of amino acids from the protein part of the original yeast flavoprotein and have also studied its combination with silver ions.

### MUTASES

Much attention has been given to the study of the mutases during the year. A full account has very recently been given by the reviewer (89).

Aldehyde mutase, the enzyme in animal tissues which catalyses the dismutation of aldehydes, has been purified and systematically studied by Dixon & Lutwak-Mann (37). It was previously believed to be identical with aldehyde oxidase (dehydrogenase) on account of the similarity of the reactions:



A being some hydrogen acceptor like methylene blue or oxygen.

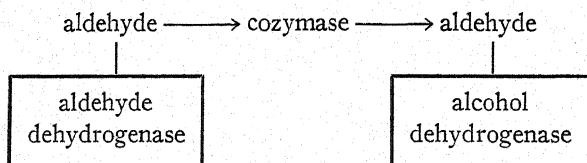
Dixon & Lutwak-Mann showed however that the two enzymes are quite different. The purified mutase has no oxidase activity, and the oxidase has no mutase activity. The mutase depends on coenzyme I, the oxidase does not. Iodoacetate completely inactivates the mutase but does not affect the oxidase; pretreatment with cyanide completely inactivates the oxidase but does not affect the mutase. The mutase was found to be absolutely specific for coenzyme I and other coenzymes were quite inactive.

As to the nature of the mutase, the fact that cozymase is necessary for the dismutation already gives some indication. It invariably acts in biological oxidations as a hydrogen carrier by being reduced by one molecule and reoxidized by another, and there is little doubt that it acts in this case by taking up hydrogen from the first aldehyde molecule and giving it up again to the second. It is clear that the mutase system contains two active centres, both of which combine with aldehyde molecules. When activated by one of the centres the aldehyde can undergo oxidation to the acid, when activated by the other it can undergo reduction to the alcohol; and the cozymase acts as a carrier of hydrogen from one to the other. The question is whether the two centres are both part of the same enzyme, in which case the mutase



would be a special enzyme of a special type, or whether they are located on two different enzymes, in which case the mutase would form a carrier-linked two-dehydrogenase system. Dewan & Green (15) have already shown that cozymase is capable of acting as a carrier in systems of the latter kind.

On the two-enzyme theory the second enzyme should of course be the alcohol dehydrogenase, which catalyses both directions of the reaction,  $\text{alcohol} + \text{Co} \rightleftharpoons \text{aldehyde} + \text{CoH}_2$ , and therefore has the required properties. The first enzyme would be a hitherto unknown aldehyde dehydrogenase capable of reacting with cozymase, and the system could be represented thus, the arrows indicating the direction of hydrogen transfer:

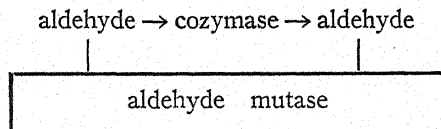


Dixon & Lutwak-Mann, however, rejected this hypothesis on the grounds (a) that no dehydrogenases could be detected in the purified mutase preparations with methylene blue and cozymase, and (b) that a system of aldehyde dehydrogenase, alcohol dehydrogenase and cozymase did not dismute aldehydes. The latter point is however not conclusive, for the known aldehyde dehydrogenase does not react with cozymase.

The significance of the first point has been modified to some extent by the later discovery of the diaphorase system, for it now appears that the failure to detect dehydrogenases was due to the fact that Dixon & Lutwak-Mann's method of purification had removed the diaphorase, which is necessary for the methylene blue reaction though not for the dismutation. Euler *et al.* (75) showed that on restoring the diaphorase alcohol dehydrogenase could be found in the purified mutase preparations, and they have again put forward the two-enzyme theory of mutase.

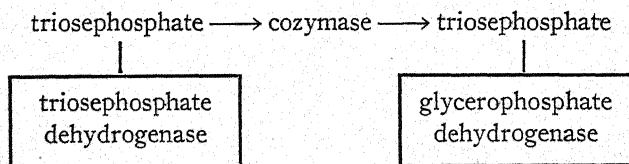
The mere presence of alcohol dehydrogenase, however, is no evidence that it has any connection with the mutase, and it is a remarkable fact that no trace of aldehyde dehydrogenase can be detected in the mutase preparations even after adding diaphorase in large amounts. This is explained by Dixon (89) on the one-enzyme theory as follows: The two aldehyde molecules are combined at neighbouring

centres on the enzyme; the oxidation of cozymase is much more rapid than its reduction in the mutase system [Green (14)]; thus when a molecule of cozymase is reduced by an aldehyde molecule on one centre it is immediately reoxidized by the other aldehyde molecule on the neighbouring centre before it has time to leave the mutase and migrate to the diaphorase; thus no reduced coenzyme reaches the diaphorase and no reduction of methylene blue is observed.



The explanation on the two-enzyme theory is not so easy, and Dixon (89) has produced other lines of evidence for the view that aldehyde mutase is a single distinct enzyme.

Following on the work of Dixon & Lutwak-Mann, Green *et al.* (35) showed the existence in muscle extract of other systems, which were at first classed as mutases because no dehydrogenases could be detected and because in one case at least ("triosephosphate mutase") a dismutation of the substrate was produced. The subsequent discovery of diaphorase revealed however that these systems consisted simply of already-known dehydrogenases, all of which could readily be detected after adding diaphorase. The "triosephosphate mutase," for example, which converts triosephosphate into glycerophosphate and phosphoglycerate, may be represented as follows:

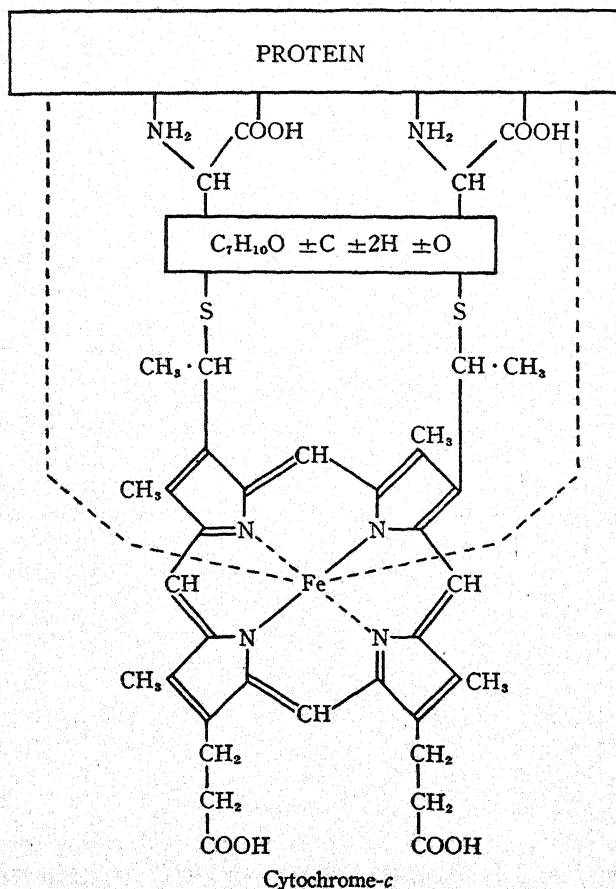


Adler & Hughes (90) have resolved it into its component dehydrogenases, neither of which alone produces the dismutation.

#### CYTOCHROME

Theorell (91) has made considerable progress in the study of the chemical structure of cytochrome-*c*. The "porphyrin-*c*" obtained by the action of acid contains side chains composed of amino acids and attached to the haematoporphyrin through two sulphur atoms. This linkage is split by hydrogen bromide in acetic acid, giving haematoporphyrin and a substance containing two sulphhydryl groups and two

$\alpha$ -amino acid groups. The latter were originally combined with the protein by peptide linkages. Thus a new type of combination of prosthetic group with protein is involved, namely through sulphur groups. Theorell suggests that cytochrome-*c* is formed by the union of the two vinyl groups of protoporphyrin with two sulphhydryl groups in the protein to form  $-\text{S}-\text{CH}-\text{CH}_3$  groups. These are not split by hydrochloric acid, peptide links being split first. Thus when the porphyrin comes away it brings a part of the original protein structure with it. It is therefore not possible to resolve cytochrome reversibly into protein and prosthetic haematin. Theorell also gives evidence that the haemochromogen-forming group is a part of the protein itself, as in haemoglobin. The structure, as far as it is known at present, is as follows:



Altschul & Hogness (92), on the basis of slight changes in absorption spectrum, have maintained, contrary to Keilin, that cytochrome-*c* forms a compound with carbon monoxide even in neutral solution. However, Keilin & Hartree (93) have shown by direct manometric measurements that this is not the case.

The physical behaviour of cytochrome-*c* when spread in the form of protein monolayers has been studied by Harkins & Anderson (94).

*Cytochrome oxidase*.—Keilin & Hartree (95) have obtained evidence that the oxidase of heart muscle does not oxidize *p*-phenylenediamine, Nadi reagent, etc., directly, but that the oxidation goes through cytochrome. The oxidase oxidizes the cytochrome and the cytochrome in turn oxidizes the reagents. Since the oxidase oxidizes only cytochrome it should no longer be called "indophenol oxidase" but "cytochrome oxidase." This work was later confirmed by Stotz *et al.* (96). Keilin & Hartree showed that the system, oxidase + cytochrome-*c* + oxygen, very rapidly oxidized catechol, hydroquinone, *p*-phenylenediamine, *o*-phenylenediamine, epinephrine, *p*-aminophenol and ascorbic acid, while without cytochrome the system had only a slight action. It is noteworthy that the cytochrome-oxidase system oxidizes ascorbic acid very rapidly ( $Q_{0_2} = 380$ ). Keilin & Hartree have also answered the criticisms of Shibata, Bigwood and others.

Observations which may have important bearings on the nature of the oxidase have recently been made by Keilin & Hartree (97, 98). It was found that the band *a* in the absorption spectrum of heart muscle is in reality due to two compounds, namely, the original cytochrome-*a*, which does not combine with cyanide or carbon monoxide and is not autoxidizable, and a new haemochromogen, now called cytochrome-*a*<sub>3</sub>, which is autoxidizable and combines with cyanide, carbon monoxide, and other respiratory inhibitors. The spectrum of cytochrome-*a*<sub>3</sub> is very similar to Warburg's photochemical spectrum of the oxidase, and as it also reacts with the respiratory inhibitors the possibility of its identity with the oxidase is suggested.

Stotz *et al.* (99) have measured the affinity with which the oxidase combines with cytochrome-*c*. The latter does not compete with the inhibitors cyanide and carbon monoxide. The inhibitors and cytochrome-*c* therefore combine with the oxidase independently at different points.

#### POLYPHENOL OXIDASES

Considerable advances have been made during the year in the study of the nature of these enzymes.

Kubowitz (100, 101, 102) purified the catechol (polyphenol) oxidase of potatoes and showed that it was a compound of copper with a specific protein. The activity was proportional to the copper content. His purest preparations contained 0.2 per cent of copper and the  $Q_{0_2}$  was 36,000, as measured by the power to catalyse the oxygen uptake of the hexosemonophosphate dehydrogenase system in presence of a little catechol.<sup>5</sup> The colour was not blue, but was slightly yellowish. On dialysing the oxidase against cyanide solution the copper was removed and the remaining protein was inactive even after removing the cyanide; but on adding a copper salt the activity returned to its original value. The oxidase can thus be split and resynthesized. Salts of other metals were inactive.

The copper is reduced by catechol and reoxidized by oxygen, and this appears to be the mechanism of the catalysis. The reduced form combines with carbon monoxide, forming a dissociable compound containing one carbon monoxide molecule to two copper atoms, and this accounts for the well-known carbon monoxide sensitiveness of this enzyme. Catechol, pyrogallol, "dopa," epinephrine and apparently *o*-diphenols generally are oxidized, but not *p*-diphenols, hydroquinone, resorcinol or ascorbic acid. On addition of a trace of catechol, hydroquinone and ascorbic acid are oxidized. Monophenols (phenol and *p*-cresol) are oxidized, but only after an induction period, and therefore probably indirectly.

Keilin & Mann (103) shortly afterwards purified the polyphenol oxidase of mushrooms, which also proved to be a copper compound but differed in several respects from the potato oxidase. Its copper content was 0.3 per cent. Its specificity was generally similar to the potato oxidase, but the pure enzyme did not oxidize monophenols. It was inhibited by cyanide, hydrogen sulphide and carbon monoxide. The activity with catechol, as measured by oxygen uptake with excess of substrate, was very high, as may be judged from the fact that at 20° one mg. copper catalysed the uptake of six litres of oxygen per minute.  $Q_{0_2}$  values with several substrates follow:

Catechol, 1,000,000; "dopa," 100,000; epinephrine, 200,000; hydroquinone, 5,000; pyrogallol, 700,000.

Very recently Keilin & Mann (104) have isolated laccase, the polyphenol oxidase from the latex of the lacquer tree. This is also a

<sup>5</sup> The oxidation product of catechol (*o*-quinone) oxidizes reduced coenzyme II directly and the catechol thus acts catalytically.

copper compound and contains 0.34 per cent copper after making allowance for a carbohydrate impurity. It differs from the other oxidases in being coloured strongly blue, in not being inhibited by carbon monoxide, and in oxidizing diamines as well as diphenols. The  $Q_{10}$ , with *p*-phenylenediamine is 40,000 and the rate with catechol is only half this. It does not oxidize monophenols (*p*-cresol, tyrosine). It is inhibited by cyanide, hydrogen sulphide, sodium azide, etc.

Dalton & Nelson (105) have recently obtained from another species of mushroom a crystalline protein containing 0.25 per cent copper, which "may be tyrosinase" (*i.e.*, polyphenoloxidase). It oxidizes catechol and *p*-cresol, but the activity is not stated.

Mann & Keilin (106, 107) have isolated from blood a pure crystalline copper-protein compound, which they call "haemocuprein." It has a blue colour and contains 0.34 per cent copper, 14.35 per cent nitrogen, 1.12 per cent sulphur. The whole of the copper of the red blood corpuscles is present in the form of haemocuprein. Its molecular weight is 35,000 and it therefore contains two copper atoms per molecule. Another copper-protein, also containing 0.34 per cent copper ("hepatocuprein") was isolated from liver, but failed to crystallize. It differed from haemocuprein in being almost colourless. Only a part of the copper of liver is in this form.

Neither of these compounds has been found to possess catalytic activity of any kind. They do not act as polyphenoloxidases, cytochrome oxidase, peroxidase, catalase, or carbonic anhydrase, and they do not combine with oxygen like haemocyanin. Their function is still unknown.

Some properties of the copper-protein compounds dealt with above are compared in Table III (p. 24).

Bhagvat & Richter (108) have carried out an extensive series of measurements on the catechol oxidase activity of tissues of different species. Apart from plants, an appreciable activity was found only in arthropods and molluscs. It is precisely in these phyla that the copper compound haemocyanin occurs and evidence was obtained that the reaction was due to its presence. The activity of the haemocyanins is small compared with that of the polyphenoloxidases, but it is sufficient to give a strongly positive phenolase test. A crystalline copper-protein was obtained from crab's blood, containing 0.13 per cent copper but differing from the haemocyanins in being colourless. With catechol it gave a  $Q_{10}$  of 16. The authors consider it to be a modified form of haemocyanin produced during the process of crystallization.

Nelson *et al.* (109, 110, 111, 112) have investigated the nature of the products of the oxidation of catechol by polyphenoloxidase (which they call "tyrosinase").

TABLE III

## COPPER-PROTEIN COMPOUNDS

Name .....	Polyphenol oxidase	Polyphenol oxidase	Laccase	Haemo- cuprein	Hepato- cuprein
Source .....	Potato	Mushroom	Lacquer latex	Blood	Liver
Cu per cent .....	0.2	0.3	0.34	0.34	0.34
Colour .....	Faintly yellow	Faintly yellow	Strongly blue	Blue	Colour- less
$Q_{O_2}$ with catechol .....	36,000*	1,000,000	20,000	0	0
Inhibition by:					
HCN, $H_2S$ .....	+	+	+	.	.
CO .....	+	+	—	.	.
Oxidizes:					
catechol .....	+	++	+	—	—
hydroquinone .....	—	—	+	—	—
<i>p</i> -phenylenediamine ..	—	—	++	—	—
<i>p</i> -cresol .....	(+)	—	—	—	—
tyrosine .....	(+)	—	—	—	—

\* Measured by a different method from the others (see text).

Szent-Györgyi *et al.* (113) have studied in detail the complexes of catechol with iron and copper and the ability of a large number of substances to enter into combination with them, and they have analysed the mechanism of the catalysis of the oxidation of catechol by metals.

## URICASE

Uricase, the enzyme which oxidizes uric acid to allantoin, has been concentrated some 500 times with respect to liver powder by Davidson (114). His purest preparations contained 0.15–0.2 per cent iron and 14.4 per cent nitrogen. As they were almost colourless the iron could not be in the form of a haematin compound. In solution at pH 10 the enzyme was stable for several weeks; it was insoluble in neutral solutions. The activity has not definitely been shown to be due to the iron, but its well-known sensitiveness to cyanide suggests that it may be a heavy-metal compound, although not inhibited by carbon monoxide. Assuming that the activity is due to the iron, 1 mg.



of iron can utilize 57 cc. of oxygen per minute in the oxidation of uric acid.

As is well-known, dogs excrete allantoin, with the exception of the Dalmatian, which excretes uric acid. The obvious assumption is that the Dalmatian is devoid of uricase. However, Klemperer *et al.* (115) found, rather surprisingly, that the liver of the Dalmatian, like that of other dogs, is rich in uricase; no correlation could be found between the amount of uricase and the amount of uric acid excreted.

#### DIOXYMALEIC OXIDASE

Banga & Szent-Györgyi (116, 117) have discovered a new oxidase occurring in many plants, which very rapidly oxidizes dihydroxymaleic acid. The authors consider it to be "One of the three basic aërobic oxidases of the vegetable kingdom, the other two being the polyphenol and ascorbic oxidases." It is not identical with either of these oxidases, and occurs in plants which do not contain them. Nor is it identical with peroxidase. Its function is not known. Hydrogen peroxide is formed in the reaction.

#### ASCORBIC ACID

*Reduction in animal tissues.*—Schultze *et al.* (118) have studied the reduction of dehydroascorbic acid in guinea pig tissues. The rapid reduction persisted after heating the tissue, and some evidence was obtained that it was due to glutathione and fixed sulphydryl groups. No evidence of any enzymic reduction was obtained.

*Reduction in plants.*—Hopkins & Morgan in 1936 found an enzyme in plant juices which vigorously catalysed the reduction of ascorbic acid by glutathione. As this reaction was more rapid than the reoxidation of ascorbic acid by ascorbic oxidase the ascorbic acid was kept in the reduced form until all the glutathione had been oxidized. Kertesz (119) has been unable to confirm the presence of this enzyme in similar juices and consequently found that in his experiments the ascorbic acid became oxidized while the oxidation of the glutathione was still in progress. However, Crook & Hopkins (120) have clearly confirmed the existence of this enzyme and studied some of its properties.

*Oxidation in animal tissues.*—Keilin & Hartree (95) showed that ascorbic acid is rapidly oxidized by cytochrome oxidase + cytochrome-c (see p. 21). Stotz *et al.* (121) obtained evidence that the oxidation in liver was due to the cytochrome system. It was inhibited



by cyanide, carbon monoxide, and sodium azide, but not by the inhibitors of copper-containing catalysts. No evidence of a specific ascorbic oxidase was obtained.

*Oxidation in plants.*—The study of the specificity and kinetics of ascorbic oxidase has been continued by Snow & Zilva (122). In addition to the ascorbic oxidase several copper-containing polyphenolase systems have been shown to oxidize ascorbic acid rapidly (see p. 21). Keilin & Mann (103) showed that highly purified mushroom oxidase would do so extremely rapidly, provided a trace of catechol was added. With 0.2 mg. of catechol, the  $Q_{O_2}$  of the ascorbic oxidation was as high as 500,000. No doubt, the catechol is alternately oxidized by the oxidase + oxygen and reduced by the ascorbic acid, thus acting catalytically. Hence the system is closely analogous to the cytochrome system. Kubowitz (102) showed that the potato polyphenolase acted similarly. Keilin & Mann (104) showed that pure laccase oxidizes ascorbic acid only after addition of a trace of *p*-phenylenediamine; catechol is inactive.

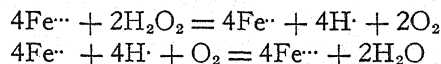
*Photochemical oxidation.*—Hopkins (123) showed that lactoflavin (or lumiflavin) acted as a photochemical sensitizer in the oxidation of ascorbic acid by oxygen. The reaction is very rapid: 2 mg. flavin in 100 cc. caused the oxidation of 160 mg. ascorbic acid in a few minutes in sunlight at room temperature. Thus we have one vitamin acting as a catalyst in the oxidation of another. They are present together in milk, and Hand *et al.* (124) later showed that in the photo-oxidation of ascorbic acid in milk the flavin is solely responsible.

Lemberg *et al.* (125, 126) have studied in detail the interesting coupled oxidation of ascorbic acid and haematins. When ascorbic acid and haemochromogen are present together a very rapid oxidation of both compounds simultaneously occurs with uptake of oxygen. The haemochromogen catalyses the usual oxidation of the ascorbic acid and undergoes at the same time a coupled oxidation to a bile pigment haemochromogen. That is to say the porphyrin ring is opened to give a bile pigment but this remains attached to the iron. The mechanism is not altogether clear, but the authors suggest that the reaction plays an important part in bile-pigment formation *in vivo*.

#### CATALASE

An important step in the elucidation of the mechanism of the catalase reaction has been made by Keilin & Hartree (127). By manometric experiments they found the remarkable fact that catalase is inac-

tive in the absence of oxygen. By spectroscopic observations in the presence of azide they were able to follow the oxidation and reduction of the iron atom of the enzyme. Catalase is of course a haematin compound, but it differs from other haematin compounds in its behaviour to oxidizing and reducing agents. The oxidized form is not reduced by the usual reducing agents, but is at once reduced by hydrogen peroxide; the reduced form is not oxidized by ferricyanide, etc., but is at once oxidized by oxygen. On the basis of these facts Keilin & Hartree put forward the following scheme of catalase action:



Thus the necessity of oxygen is explained: its function is to oxidize the iron, which in turn oxidizes the hydrogen peroxide to oxygen. It will be noticed that this mechanism is very similar to that of the polyphenoloxidases, hydrogen peroxide taking the place of the catechol. It seems in fact that we may almost consider catalase as an oxidase of hydrogen peroxide.

The molecular weight of crystalline catalase from liver (128) has been studied in the ultracentrifuge by Stern & Wyckoff (129, 130), who obtained a value of 250,000 to 300,000, and by Sumner & Gralén (131, 132), whose latest value is 248,000. It contains four haematin groups per molecule.

Agner (133) has obtained highly purified liver catalase preparations which were more than twice as active as the crystalline catalase. They contained small amounts of copper as well as iron. In the ultracentrifuge they were found to consist of a protein of molecular weight 225,000, which was shown to contain 0.1 per cent iron but no copper, together with about 15 per cent of another protein of a different molecular weight, containing 0.16 per cent copper but no iron. The connection of this with catalase activity is not clear.

#### THE CITRIC ACID CYCLE: CATALYSIS BY THE C<sub>4</sub> ACIDS

Krebs & Eggleston (134) have found that insulin can produce a large increase of oxygen uptake by minced muscle, but only when citrate or boiled muscle extract (or better still, both) are also added. They consider that insulin acts as a catalyst in connection with one of the stages of the citric acid cycle (135).

Krebs *et al.* (136) have confirmed earlier findings that citric acid

is excreted in the urine after injection of the  $C_4$ -dicarboxylic acids (succinate, fumarate, malate, oxalacetate) and regard this as evidence in support of the citric acid cycle. Smith & Orten (137) find that the rate of this citric excretion reaches a maximum about one hour after the injection. They suggest that the citric formation takes place in the kidney.

Breusch (138), on the other hand, has strongly criticized the evidence for the existence of the citric acid cycle, at any rate in muscle. He states that the essential step in the cycle, namely the formation of citrate from oxalacetate, does not occur in the tissues studied. The citrate produced on incubating oxalacetate with muscle in Krebs' experiments was formed, not from oxalacetate, but by the action of the tissue on an artificial condensation product formed by neutralizing oxalacetic acid in concentrated solution. When the oxalacetic acid is diluted before, instead of after, neutralization, no citrate is produced on incubation with muscle. Moreover no citrate is produced from fumarate or malate, although it should be formed if the cycle occurs. Oxalacetate is not converted by the muscle into citrate but into malate, in accordance with Szent-Györgyi's scheme of respiratory catalysis by the  $C_4$  acids. Breusch believes that Szent-Györgyi's system, rather than the citric acid cycle, accounts for the respiration of muscle.

There is now good evidence for the catalysis of respiration by the  $C_4$  acids in many animal tissues, but Breusch (139, 140) finds that it does not occur in lung or in mouse tumour, owing to a complete lack of the ability to reduce oxalacetate. Califano & Banga (141) have also found that the  $C_4$  acids do not act catalytically in *Esch. coli*.

#### OTHER CELL OXIDATIONS

The oxidation of alcohol by animal-tissue slices has been studied by Leloir & Muñoz (142), who find that liver is the only tissue in which the process is at all rapid. The product is acetic acid. The oxidation is greatly accelerated by pyruvate, and is inhibited by cyanide and by iodoacetate.

Bernheim (143) has found a remarkable accelerating effect of alloxan on the oxidation of alcohol by liver. Under certain conditions 0.0002 *M* alloxan may produce a thousand per cent increase of the rate of oxidation. Alloxan had no effect on the oxidation of a number of other substances by the liver. The effect is not yet explained.

Clark *et al.* (144) find an effect of insulin on the oxidation of alcohol by liver. The livers of depancreatized, diabetic cats lost the

power of oxidizing alcohol almost completely, whereas the livers of depancreatized, insulin-treated cats (non-diabetic), or of diabetic cats after an injection of insulin, had the normal oxidizing power.

Bernheim & Bernheim (145) found that a large extra oxygen uptake by liver suspensions was produced by adding a few  $\mu\text{g.}$  of vanadium. This was due to a catalytic action of the vanadium on the oxidation of a substance—probably a phospholipid—which was extracted from the tissue but not identified. Bernheim *et al.* (146) have shown that pyrrole considerably accelerates the oxidation of *d*-amino acids and amines by liver and kidney preparations, particularly in the presence of a little methaemoglobin.

Barron & Lyman (147) have studied further the catalysis by haematin compounds of the oxidation of unsaturated fatty acids. They find among other things that cyanide inhibits the catalysis by haemochromogens but not by haematin.

Franke & Jerchel (148) have made a study of peroxide formation during the autoxidation of unsaturated fatty acids.

The course of the oxidation of acetaldehyde by yeast has been investigated in detail by Heicken (149).

#### NOMENCLATURE

The terminology of the subject is at the moment in an unsatisfactory state, largely owing to the coexistence of several different systems of nomenclature adopted by different schools. The Warburg school, in particular, has adopted a somewhat unorthodox terminology [see Warburg (150)], which has not been accepted by the majority of workers elsewhere. It is now extremely difficult, without an intimate knowledge of the subject, to bring the facts discovered by the different schools into relation with one another. The fundamental nature of the divergence may be seen for instance by comparing Kubowitz's (84) statement, that one may build up an alcohol dehydrogenase from diphosphopyridine-proteid, catechol and copper proteid, with the same fact expressed in the terminology used by others, namely that a complete oxidase system may be formed from alcohol dehydrogenase, cozymase, catechol and catechol oxidase.

It is not the intention of the reviewer to discuss here the merits of the various systems, but it may be helpful to give a list of terms with their equivalents (Table IV, p. 30). It is to be noted that the "proteins" of Warburg, in the case of dehydrogenases at any rate, correspond closely with the ordinary conception of enzymes. It is they which

contain the "active centres," entering into specific combination with the substrate and activating it. The specificity and nature of the reaction are determined entirely by the "protein" component. The so-called prosthetic groups act simply as intermediary hydrogen acceptors, taking up hydrogen from the activated substrate and passing it on to other molecules.

TABLE IV  
NOMENCLATURE

Warburg School	Others
Protein	Enzyme (especially dehydrogenase) [Apo-enzyme (Euler)]
Enzyme (ferment)	{ Enzyme + coenzyme [Holo-enzyme (Euler)] or protein + prosthetic group ( <i>e.g.</i> , flavoprotein)
Dehydrase	Complete oxidase system (usually containing several enzymes)
Partner	Substrate
Respiratory enzyme	{ Cytochrome oxidase
Autoxidizable iron-proteid	
Yellow enzyme	{ Flavoprotein
Alloxazine-proteid	
Pyridine-proteid	Dehydrogenase + coenzyme I or II
Copper-proteid	Polyphenol oxidase
Protein of aldehyde reductase	{ Alcohol dehydrogenase
Protein <sub>Alcohol, Aldehyde</sub>	
Protein of Negelein	
Intermediary enzyme ( <i>Zwischenferment</i> )	Hexosemonophosphate dehydrogenase
Alloxazine-adenine- proteid <sub>oxygen, amino acids</sub>	Amino acid oxidase + coenzyme
Diphosphopyridinenucleotide	Coenzyme I, Cozymase
Triphosphopyridinenucleotide	Coenzyme II

#### POTENTIALS

The oxidation-reduction potential of cytochrome-*c*, about which there was some disagreement, has been redetermined by several workers. Wurmser & Filitti-Wurmser (151, 152) found by direct electrode measurements that the value of  $E'_0$  was constant at +0.253 v. between pH 5 and 8 at 25°. Equilibrium was only reached after four to five hours. This value was confirmed by studying the equilibrium between

cytochrome and reductinic acid, the potential of which was already known. The cytochrome potential satisfactorily obeys the equation

$$E_h = E'_0 - \frac{RT}{F} \log_e \frac{[\text{reduced cytochrome}]}{[\text{oxidized cytochrome}]}$$

Stotz *et al.* (153) obtained the value  $E'_0 = +0.262$  v. between pH 5 and 8 at  $30^\circ$  by spectrophotometric measurements of the equilibrium between cytochrome and certain dyes of known potential. Laki (154), by spectroscopic observations on the equilibrium of cytochrome-*c* in washed muscle with quinhydrone, obtained  $E'_0 =$  about  $+0.280$  v. at pH 7.4. Ball (155) similarly obtained  $E'_0 = +0.27$  v. at pH 7.4 and  $20^\circ$ .

These results, which were all obtained on muscle cytochrome, are in fairly good agreement, considering the diversity of methods used and the conditions. They differ considerably from Green's (156) previous value ( $E'_0 = +0.127$  v.), which, however, was obtained with yeast cytochrome-*c*.

Ball (155), by spectroscopic observations on heart muscle suspension in presence of various dyes, quinone, ferricyanide, etc., has deduced approximate values for the  $E'_0$  of the three components of cytochrome at pH 7.4 and  $20^\circ$  as follows: cytochrome-*a*,  $+0.29$  v.; cytochrome-*b*,  $-0.04$  v.; cytochrome-*c*,  $+0.27$  v.

Clark (157) has calculated the potential of coenzyme I from its equilibrium with the lactate/pyruvate system, the potential of which was already known. He finds that at pH 7.4 and  $20^\circ$   $E'_0 = -0.325$  v.

Michaelis & Schwarzenbach (158) have continued the study of the potentials of flavins and the conditions of semiquinone formation, and semiquinone formation in other systems has been studied by Michaelis *et al.* (159, 160) and by Hill (161).

Wurmser & Filitti-Wurmser (162) have determined the potential of the system  $d(-)\text{-alanine} \rightleftharpoons \text{ammonium pyruvate}$  in presence of the *d*-amino acid oxidase of kidney, using Nile blue as carrier. They find  $E'_0 = -0.066$  v. at pH 7.3 and  $37^\circ$ .

Hoff-Jørgensen (163) has investigated the system  $\beta$ -hydroxybutyrate  $\rightleftharpoons$  acetoacetate in presence of the dehydrogenase from heart, with cozymase and phenosafranin. At pH 7  $E'_0 = -0.2931 \pm 0.0001$  v., and between pH 6 and 7.8 the  $E'_0$ :pH curve had the 60 mv. slope. For this reaction

$$\Delta F_{38^\circ} = -6399 \pm 5 \text{ cal.}, \text{ and } \Delta H_{38^\circ} = -16890 \pm 100 \text{ cal.}$$

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# PROTEOLYTIC ENZYMES

BY K. LINDERSTRØM-LANG

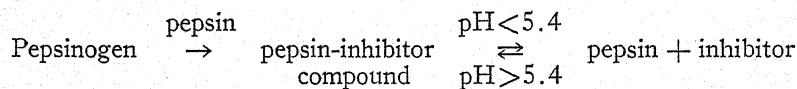
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## CHEMISTRY

### PREPARATIVE AND CONSTITUTIONAL CHEMISTRY

*Pepsin*.—In an extensive paper, Herriott (1) has described the isolation, crystallization and properties of swine pepsinogen [compare (2)]. The method deviates somewhat from others applied in Northrop's laboratory inasmuch as an adsorption on copper hydroxide and elution with phosphate buffer, pH 6.8, was introduced prior to the final precipitation and crystallization with ammonium sulphate. The crystals obtained were fine needles which, under the condition studied, seemed to contain only one protein component.

Apart from being catalytically inactive, pepsinogen differs from pepsin in crystalline form, isoelectric point [3.8 instead of 2.7 (see below)], amino nitrogen and several other properties among which limits of stability, reversibility of denaturation may be mentioned. The activation to pepsin is essentially an autocatalytic process in which a portion of the pepsinogen molecule containing 15 to 20 per cent of the pepsinogen nitrogen is split off. Approximately nine peptide bonds per molecule are opened. The process is however complicated by the circumstance that the compound (or one of the compounds) split off from pepsinogen acts as an inhibitor for the pepsin formed (3). Herriott gave the following scheme:



pK for the equilibrium of the latter reversible reaction is 5.4. The inhibitor is unstable to alkalis (like pepsin). It is split by pepsin, is soluble in boiling 2.5 per cent trichloroacetic acid and dialyses slowly through collodion membranes.

That the activation of pepsinogen is an ordinary proteolytic reaction with no "intrinsic" specificity is shown by the fact (4) that swine pepsin transforms chicken pepsinogen into chicken pepsin and *vice versa*.

Several experiments have been carried out to test the individuality of pepsin. Tiselius, Henschen & Svensson (5) studied the electro-

phoresis of pepsin in the Tiselius apparatus and found that a portion of dissolved crystalline pepsin migrated as an active, homogeneous protein. Some inactive protein material was left behind in quantities varying from 20 to 50 per cent of the total [compare (6)]. Unfortunately the ratio of activity (hemoglobin method) to total nitrogen of the migrating protein varied between rather wide limits, from 0.25 to 0.34, with the pepsin preparation employed. The reason for this is as yet obscure. It cannot be due to the presence of the above-mentioned inhibitor since this does not combine with pepsin within the pH-range investigated by Tiselius *et al.* The possibility exists that we shall have to look for a pepsin activator. Another peculiar fact found was that pepsin is negatively charged at all pH values between 1 and 4.4. This confirms some older experiments by Ringer and by Northrop but disagrees with more recent determinations by Northrop (7). The explanation given by Tiselius *et al.* is that the pepsin molecules combine with negative ions in strongly acid solutions, their own charge being masked in this way.

In another manner Steinhardt (8) has strengthened the view that some crystalline enzymes may be mixtures of protein material. He has studied the solubility of several proteins, including crystalline pepsin, and found the typical dependence upon the quantity of solid phase which led Sørensen (9) to consider certain proteins to be "reversibly dissociable component systems." The reason for this behaviour of pepsin is not likely to be found in a dissociation of pepsin itself—pepsin does not dissociate in urea for example (10)—but is no doubt connected with the above-mentioned findings that the crystalline material is not homogeneous. Several investigations from Northrop's laboratory tend to show that the proenzymes behave more like individual substances in this respect than the enzymes derived from them [compare (11) and (1)].

The repeated observation [compare (12)] that beautifully crystalline enzyme preparations may contain large quantities of impurities is a warning to the enzyme chemist. We cannot always expect the impurities to be catalytically inactive. The solubility test is not in every case a reliable test for homogeneity, and careful and varied investigation of each substance must decide the question of its individuality.

An interesting physicochemical study of pepsin may be mentioned, *viz.*, the study of the activity of pepsin monolayers by Langmuir & Schaefer (13) [compare (14)]. They found that such monolayers

spread on water and then deposited on plates, or adsorbed from solution by conditioned plates showed high enzymatic activity when dissolved off from the plates by the substrate (skim milk).

As regards the chemistry of pepsin, Philpot & Small (15) studied the action of nitrous acid on pepsin under conditions where no damage is done to the protein structure in general (acetate buffer, pH 3.8 to 4.5). It is a simple reaction which is much retarded when pepsin has lost 50 per cent of its activity. Taking into account similar experiments with tyrosine (16) the authors arrived at the conclusion that the tyrosine groups of pepsin are involved in the process [compare (17)] and it appears that half of the tyrosine groups per molecule have reacted when the activity has fallen to half. This result shows with perfect clearness that the tyrosine groups participate in the enzymatic reaction with peptide bonds (17), but leaves unknown how they participate. The most obvious explanation would be that pepsin is a "multi"-enzyme with many active centers, *viz.*, certain sets of tyrosine groups (1, 2, 3...). It is however inconceivable in this case how the residual tyrosine groups, which do not react with nitrous acid, can have the same activity per group (or set) as the others. It may of course be a mere coincidence that the disappearance of half of the tyrosine corresponds to a loss of half of the activity [see (15)], but it is reasonable then to assume that the residual tyrosine groups have no connection with the remaining activity; in other words, one may anticipate that pepsin has a double specificity, bound to two different kinds of groups. An investigation of the specificity of diazopepsin would elucidate this question. An alternative explanation would be that the participation of the tyrosine groups in the proteolytic process is an indirect one, due either to their function in the general structure of the protein molecule or to some intermediary function, important for the capture or transport of specific substances, ions or water molecules necessary for the catalytic reaction and capable of penetrating into the protein molecule.

*Chymotrypsin, trypsin.*—An important case of gradual enzyme transformation without actual change in catalytic activity was described by Kunitz (12). If chymotrypsin which crystallizes in rhombohedrons or prisms is left to stand in aqueous solution at 5° and pH 7.6 to 8.6 it is transformed into a mixture of three proteins which will crystallize together in fine needles. Two of the three proteins are active ( $\beta$  and  $\gamma$  chymotrypsin), one is inactive. The  $\beta$  and  $\gamma$  chymotrypsin may be separated from the inert material and from each other



and crystallized. They are almost identical with the original chymotrypsin in catalytic activity but they differ from it and from each other with regard to crystal form, molecular weight, solubility, titration curves and stability in acid, alkali and urea.

The differences in molecular weight are remarkable: original chymotrypsin, 40,000;  $\beta$  chymotrypsin, 30,000; and  $\gamma$  chymotrypsin, 27,000. This may be regarded as important chemical evidence against the validity of the cyclol theory (18) for crystalline proteins in general. If chymotrypsin has the structure  $C_2$  (18) the  $\beta$  and  $\gamma$  forms cannot possibly have the same structure.

A further contribution by Kunitz (19) deals with the activation of chymotrypsinogen and trypsinogen by an enzyme from *Penicillium*. The enzyme has the molecular weight 40,000 and is alkali-unstable. It transforms trypsinogen into trypsin at about pH 3.5 without loss of protein from trypsinogen. Chymotrypsin is activated much more slowly.

Schmitz (20) described a trypsin inhibitor in blood. It is soluble in water and solutions of trichloroacetic acid but may be precipitated by ammonium sulphate. It does not inhibit chymotrypsin [compare (21), (22), and (23)].

*Papain*.—Balls, Lineweaver & Thompson, (24) have added this enzyme to the list of crystalline proteases. The crystals are obtained from the coagulated latex of *Carica papaya* by fractional precipitation with ammonium sulphate and crystallization from very concentrated solutions. They are fine needles or—when slowly formed—beautiful hexagonal crystals (private communication). Without added activator the activity (per mg. of protein nitrogen) varied considerably from one preparation to another. Treatment with cystein eliminated the variations, however, and increased the activity from 25 to 50 per cent above that of the best amorphous preparations. It is important to note that the ratio

$$\frac{\text{rate of casein digestion}}{\text{rate of splitting of benzoylglycine amide}}$$

was the same for the crystalline material as for the dried latex and for amorphous preparations (see below). Kassell & Brand (25) have determined the sulphur distribution in papain but since the enzyme sample used was not pure the figures may need revision. They found that the sample analyzed contained 1.47 per cent sulphur, 76 per cent

of which was attributable to cystine + cystein; 7 per cent to methionine; and 17 per cent was not accounted for.

The question of the natural activation of papain was studied by Frankel, Maimin & Shapiro (26, 27) and by Ganapathy & Sastri (28, 29). Glutathione is without doubt present in the latex (26, 29) but to say that glutathione is the natural activator is somewhat unwarranted. The idea that papain is activated by glutathione as a specific activator (zookinase, phytokinase) originated before the activation was considered to be a reduction. If this more modern conception is adopted, *i.e.*, activation by reduction, there is no special reason to exclude the possibility that papain, like glutathione, is able to enter directly into the metabolic system of the plant and become reduced by that system.

According to Morgan & Friedmann (30) maleic acid acts as an inhibitor to activated papain. Jørgensen (31) has shown that bromate and iodate both show strong inhibition in contrast to chlorate which has no effect. According to Bergmann & Fruton (32) the finer differences in the specificity of papain are highly dependent upon activation by cystein and phenylhydrazin (see below).

*Peptidases.*—As regards the preparative and constitutional chemistry of peptidases no important papers have appeared in 1938 except: a general review on crystalline carboxypeptidase [Anson (33)] in which the isolation and properties of this enzyme and its precursor are described; and a contribution on the separation of dipeptidase and aminopolypeptidase [Abderhalden (34)]. Grassmann, Volmer & Windbichler (35) reported some interesting attempts to separate yeast dipeptidase into a coenzyme and an apoenzyme. Leucylglycine was used as a substrate. No reference was made however to the work by Johnson, Johnson & Peterson (36) on the magnesium activation of leucylpeptidase [compare (37, 38)] and it is a question whether the more important of their findings could not be explained as a magnesium activation.

*Arginase.*—The activation of arginase was studied by Hellerman & Stock and the results obtained reported in an extensive paper (39). Interesting pH curves were found for the action of arginase "alone" and with addition of cobalt, nickel and manganese ions. The pH-optimum was strongly displaced to lower pH values by cobaltous and nickelous ions, and to higher pH values by manganous ions. Edlbacher & Baur (40) reported that dialysis of yeast and liver arginase resulted in complete inactivation. The activity was restored by adding manga-

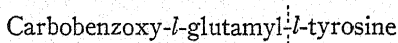
nese, cobalt, nickel, cadmium and vanadium ions, the manganese ions being by far the most effective, acting even in such low concentrations as  $3 \times 10^{-6} M$ .

*Urease.*—The molecular weight of urease was determined by Sumner, Gralén & Eriksson-Quensel (41) and found to be 483,000, a very high value indeed compared even with that of catalase (250,000). In connection with the above-mentioned inhomogeneity of pepsin it is interesting to note that the best preparation of urease, when analysed in the centrifuge, contained 15 per cent impurity.

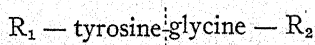
Gorter & Maaskant (42) studied the spreading of urease and found an isoelectric point at pH 5.0. Stern & Salomon (43) were unable to demonstrate any formation of an enzyme-substrate compound by spectrographic means. Pillemer, Ecker, Myers & Muntwyler reported that the inactivation of urease by irradiation is essentially irreversible (44).

#### CHEMISTRY OF ACTION

*Breakdown.*—An important contribution to our knowledge of the action of pepsin is the discovery by Fruton & Bergmann (45) of a synthetic substrate for this enzyme:

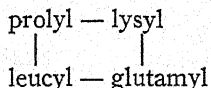


Hence pepsin acts as a carboxypolypeptidase in this special case. The residues of glutamic acid and of tyrosine must both be significant for the enzymatic fission of the peptide bond marked with the vertical line. It must be remembered that the action of pepsin has been tested several times on a great number of polypeptides containing mono-amino-mono-carboxylic acids only (including tyrosine) and always with negative results. We now see how liberation of tyrosine [Calvery, Block & Schock (46, 47); Damodaran & Krishnan (48)] can occur in peptic digestion but it seems evident (48) that only a small portion of the tyrosine is bound in such a way in the protein molecule that free tyrosine will be liberated by the action of pepsin alone [compare Abderhalden (49)]. It would be an interesting experiment to compare the amount of free tyrosine liberated from proteins by pepsin alone with that liberated by chymotrypsin followed by pepsin since it was found that chymotrypsin [Bergmann & Fruton (50)] would split bonds of the following kind



Another substrate for pepsin was reported by Tamura (51) who found that diglycyl-diaminosuccinic acid was split by this enzyme, and by trypsin, aminopolypeptidase and papain as well. Yeast dipeptidase had no effect. It appears that one of the glycine residues in the meso-form of this substrate is split off completely by pepsin in the course of 24 hours (under the experimental conditions chosen). This disagrees so entirely with generally accepted views on the action of pepsin that co-operation from other laboratories is needed. Moreover the question of the action of proteolytic enzymes on diketopiperazines has been taken up again by Tamura (51). The hydrolysis of glycylaspartic acid anhydride and aspartic acid anhydride by a glycerine extract of pancreas was investigated and a small amount of cleavage observed. The quantity of enzyme used however was very large and this may have introduced some uncertainty into the results. It is recommended that in such publications all or at least some control experiments (blanks for self-digestion of enzyme, blanks for nonenzymatic hydrolysis of substrate, etc.) be reported in detail especially when the results are so very important to our understanding of the action of proteolytic enzymes. How decisive such control experiments may be was shown by Abderhalden & Leinert (52), who were able to demonstrate that the breakdown of *l*-histidine anhydride in acid solution was quite independent of the presence of pepsin. [Compare Shibata (53)].

In connection with the review of the endeavours to find synthetic substrates for pepsin a paper by Fodor & Kuk (54) may be mentioned dealing with the splitting of "akropeptides" by pepsin, papain and aminopolypeptidase. The paper is one of a long series [compare (55)] on the degradation products (akropeptides) formed from gelatine and casein in hot glycerine, and in spite of the fact that the experimental proof of the structural formulae presented is insufficient the work is suggestive and deserves being tested in other laboratories. What awakens the interest of the reader is, for example, that a supposed tetrapeptide ring present in an akropeptide from casein



is reported to be split by pepsin and papain. By the action of one of these enzymes the ring is opened and the peptide chain formed may be split by aminopolypeptidase.

Bergmann & Fruton (50) have published an interesting study of the action of chymotrypsin on synthetic substrates as compared with that of papain-HCN [see also Bergmann & Fruton (56)]. Some of the results may be summarized in the following table:

Substrate	Chymo- trypsin	Papain- HCN
Carbobenzoxyglycyl- <i>l</i> -tyrosyl-glycine-amide <i>pap. chym. pap.</i>	+	+
Benzoyl- <i>l</i> -tyrosyl-glycine-amide <i>chym. pap.</i>	+	+
Benzoyl- <i>d</i> -tyrosyl-glycine-amide	—	—
Benzoyl- <i>dl</i> -tyrosyl-glycine-amide	—	—
Benzoyl- <i>l</i> -tyrosine-amide <i>pap.</i>	—	+
Benzoyl- <i>d</i> -tyrosine-amide	—	—
Benzoyl- <i>dl</i> -tyrosine-amide	—	—

The difference in the mode of action of the two enzymes is clearly seen from this table. But some other features of general nature were also observed. Although benzoylglycine amide is split by papain with formation of benzoylglycine and ammonia, benzoyl-*d*-tyrosylglycine-amide is not attacked, showing the inhibitory effect of the distant *d*-tyrosine residue. It is interesting to compare this result with the finding by Waldschmidt-Leitz & Balls (57) that both *d*- and *l*-leucyl-glycyl-*l*-tyrosine are split by carboxypolypeptidase.

The negative results of the experiments with the racemic compounds are indeed very instructive. They are not due to an inhibition of the splitting of the *l*-compound by the *d*-compound but appear to be due to a formation of a racemate, stable in aqueous solution. They teach us to be very cautious in using racemic compounds in enzymatic investigations.

From the literature of the past three years, especially from the papers from Bergmann's laboratory, something like a picture of the proteinase action is beginning to take form. The idea that the side

chains of certain amino acids are able to direct the proteinases in their choice of peptide bonds seems to be borne out by all investigations. Moreover the above-given comparison between chymotrypsin and papain shows that differences in specificity may depend upon which of the two groups in an amino acid—the  $\alpha$ -amino or the carboxyl group—is involved in the peptide bond attacked. Thus eventually we may distinguish between a tyrosine-amino-endo-peptidase (present in papain) and a tyrosine-carboxy-endo-peptidase (identical with or present in chymotrypsin). Whether such a classification is justified cannot be decided yet. We know that clupein is rapidly hydrolyzed by papain and that sturin (58) is split by chymotrypsin in spite of the fact that this protamine does not contain tyrosine; benzoylglycine amide (see above), benzoylarginineamide and carbobenzoxy-iso-glutamine are split by papain under certain conditions (32). We must therefore be ready to face the possibility either that both enzymes are mixtures of several enzymes or that their range of specificity is broader than indicated above. After all the directing influence of the side chains may manifest itself mainly in a labilization of adjacent peptide bonds and thus be of less specific nature [compare (59)]. The mechanism of such a labilization, a sensitization to the proteinase attack, is yet obscure but may consist in influencing the electron displacement at the peptide band [compare Taylor (60) and Hinshelwood *et al.* (61)]. On the other hand the proof of the individuality of an enzyme is extremely difficult to furnish and nature may have great surprises in store for us.

The elementary picture of proteinase attack seems to become clearer each year. It is essentially an opening up of the peptide bonds in the middle of either open or closed peptide chains, *i.e.*, in compounds simulated by the above-mentioned synthetic substrates. There is hardly any doubt that in the case of a number of substances classified among the proteins—gelatine, protamines or even casein—this fission of peptide bonds is the initial and sole reaction taking place in proteolytic hydrolysis. In other cases, however, it is evident that a secondary structure in the protein molecule or micella blocks the peptide bonds to a certain degree and that a different—enzymatic or non-enzymatic—initial reaction must take place prior to the fission of peptide bonds. Such cases are found in the crystalline or globular proteins and in sclero-proteins. The blocking may have its origin in steric hindrances or in the presence of chemical structures into which the peptide bonds have entered themselves [*e.g.*, cyclol formation (Wrinch)]. Finally

the presence of specific inhibitors [compare (23)] may sometimes be important.

Experiments purporting to show initial enzymatic reactions, other than fission of peptide linkages, have often been reported (enzymatic disaggregation without breaking of peptide linkages [compare (62) and (63)], denaturase action, etc.) but the existence of such reactions has never been satisfactorily proved. Mention may however be made of an interesting finding by Gorter, Maaskant & Van Lookeren Campagne (64) which indicates that trypsin has an explosion-like action on the spreading of fibrinogen. The importance of this phenomenon to the transformation of fibrinogen into fibrin (blood coagulation) is obvious, but nothing is known about the chemical process involved.

Some non-enzymatic, initial reactions preparing the protein molecule for the attack of proteinases are known.

Initial Reactions	Substances Affected
Reactions occurring at high acidity	Several proteins
Heat denaturation	Several proteins
Reduction in alkaline medium	Keratin
Reaction with ammoniacal copper hydroxide solution	Fibroin (65)
Mechanical disintegration (ball mill)	Keratin (66), collagen (67)

Linderstrøm-Lang, Hotchkiss & Johansen (68) have further called attention to the possibility that reversible denaturation may be the initial reaction in the proteolytic breakdown of genuine globular proteins in neutral solution. They have warned against the conclusion that these proteins, when genuine, contain peptide bonds because they are split for example by trypsin under conditions where they are stable in the absence of biocatalysts. In general it may be feared that the complex structure of these proteins is not open to study by enzymatic analysis.

Using deuterium as an indicator Stekol & Hamill (69) reported that the hydrogen atom bound to the  $\alpha$ -carbon atom in tyrosine became labile during the splitting off of this amino acid from proteins by enzyme action. Foster, Keston, Rittenberg & Schoenheimer (70), in a careful investigation of the tryptic digestion of casein, were unable to reproduce this result.

According to Damodaran & Ananta-Narayanan (71) ammonia is not liberated from casein, edestin and gliadin by the action of pepsin, trypsin and erepsin (absence of amidases).



The specific milk-clotting power of many proteolytic enzymes was studied by Balls & Hoover (72) and by Lundsteen (73). The mechanism is unknown [compare the previously mentioned paper by Gorter *et al.* (64)] and may be different in the case of different enzymes. The distinct differences in pH optima (73) are suggestive.

Abderhalden & Hanson (74, 75) have found that carboxypeptidase isolated according to Anson shows some dipeptidase activity which may be suppressed by addition of trypsin or formaline or by heating to 56°. They suggest that some reaction involving sulphhydryl groups takes place in the former cases. According to Purr (76), glutathione (coenzyme for methylglyoxalase) is split by a special enzyme (iminopolypeptidase) acting as antiglyoxalase. The enzyme is different from the enzymes splitting leucylglycylglycine and chlor-acetyltyrosine.

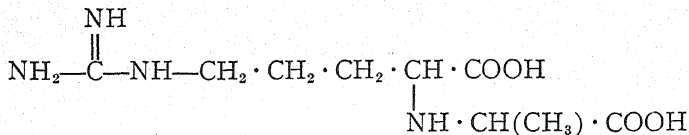
Schneider (77) tested the activity of dipeptidase and aminopolypeptidase toward peptides containing aminomalonic acid and diaminopropionic acid. Practically all combinations of these amino acids with glycine and ammonia were split by yeast dipeptidase. The reported fission of *l*-alanyl-aminomalonic acid diamide by dipeptidase is incomprehensible in view of the generally accepted theory that a free carboxyl group is necessary for the action of this enzyme. No tests for liberation of ammonia were reported.

In a reinvestigation of previous work on specificity Johnson (78) studied the intestinal aminopolypeptidase and reported that prolyldi-glycine and polypeptides in which one hydrogen atom in the terminal amino group is replaced by alkyl are split by this enzyme. He expressed the opinion that there is no reason to assume the presence of a specific prolinepolypeptidase [compare (79)]. Abderhalden & Abderhalden (80) on the other hand reported experiments incompatible with this view. Here again we meet the difficulty of proving or disproving the individuality of an enzyme.

In an interesting contribution to the theory of peptidase action, Berger, Johnson & Peterson (81) have found that the peptidases of *Leuconostoc mesenteroides* are able to hydrolyze both optical isomers of di- and tripeptides. The effects are much greater than the possible experimental error, but because of the extreme importance of this observation the isolation of the *d*-amino acids in question from the reaction mixtures would be valuable additional evidence.

The splitting of tetrapeptides built up of glycine, alanine and tyrosine was investigated by Abderhalden *et al.* (82).

Asaki (83) studied the splitting of octopin



and its isomer by arginase and found the pH-optimum at 9.5. Hunter (84) found that carbamidoarginine was slowly hydrolysed by arginase, pH-optimum 9.7. Felix, on the other hand, maintains that the optimum for the splitting of all substrates other than arginine (even  $\epsilon$ -guanidino- $\alpha$ -aminocaproic acid?) is at about pH 7 [compare (85)]. Hellerman & Stock (39) have pointed out the possibility that divergencies concerning pH-optima may arise from uncontrolled influences by ions (cf. pp. 41-42).

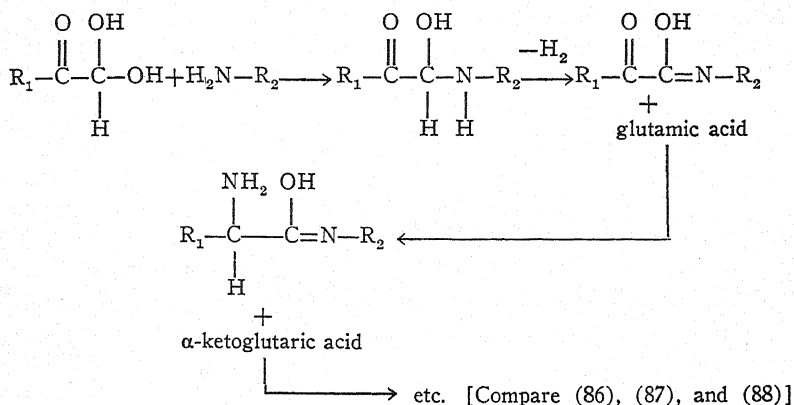
The specificity of arginase is still in question. All investigators agree that substitution in the guanido and carboxyl groups completely blocks the enzymatic action, but there is considerable disagreement concerning substitution in or removal of the  $\alpha$ -amino group. Thus Felix & Schneider (85) reported that argininic acid ( $\epsilon$ -guanido- $\alpha$ -hydroxyvaleric acid) is split by arginase and that the reaction is activated by manganese ions. Similar experiments by Hellerman & Stock were negative. One thing, however, seems to be certain, namely, that arginase acts slowly and incompletely on all substrates other than arginine itself. [Concerning the theory of action of arginase see (39).]

### SYNTHESIS

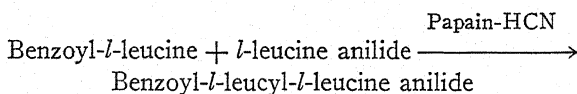
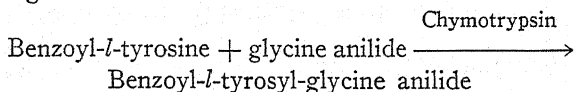
Our knowledge of protein synthesis in the living cell has increased slowly and we must admit that we have no definite ideas concerning this question. We have every reason to believe that hydrolytic enzymes isolated from cells or cell secretions may, under suitable conditions, effect syntheses, *i.e.*, reverse hydrolysis, but we do not know whether they are actually used by the cell for such synthetic purposes. Synthesis may, in the cell, pass through intermediate metabolic steps which lie partly or entirely outside of the range of action of the known proteolytic enzymes.

In order to make this clear but with no intention to set forth any definite hypothesis the following scheme is presented to show how peptide chains may be built up in reactions closely connected with the general metabolism of the cell but apparently without co-operation

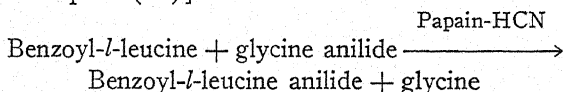
of proteases in the usual sense (or with the mode of action generally "assumed").



In continuation of previous work Bergmann, Fruton, Fraenkel-Conrat & Behrens (50, 89, 90) have studied the simple reversal of proteolysis. The necessary fall in free energy in the direction of synthesis was produced by the continuous removal of the insoluble synthetic products by crystallization. Several very interesting cases of synthesis were reported using both chymotrypsin and papain as catalysts, e.g.



A type of process was also described (89) in which no formation or disappearance of amino and carboxyl groups occurs ["peptide transmutation," compare (91)]:



An old case of synthesis, but actually of exactly the same general type as those given, *viz.*, the plastein formation by pepsin, was studied by Kumamoto (92). Kumamoto separated from ovalbumin peptone a fraction, soluble in absolute alcohol (?), having the ratio of primary amino nitrogen to total nitrogen equal to one-third, and showing a

marked synthesis (*vis.*, a formation of a precipitate!) in concentrated solution. The optimum of the synthesis was at pH 4. After isolation the precipitate was split by pepsin. Unfortunately several important control experiments were omitted.

The mechanism of the synthesis of protein in the living cell is very obscure and can only be clarified in connection with the study of coupled reactions in the metabolic system. Several attempts to imitate this protein formation (*in vitro*, *vis.*, under simpler conditions) have been reported (93, 94, 95, 96, 97) but no definite results have been obtained [compare (98)]. The problem is one of the most fascinating ones but cannot be dealt with in detail here. Two important sources of error in such investigations may be just mentioned: The reaction of amino groups with aldehydes (sugars, etc.) causing a drop in amino nitrogen (Van Slyke method) [compare (99, 100, 101, 102); for further literature see (102)]; and the formation of intermolecular disulphide linkages between peptides giving rise to substances precipitable by trichloroacetic acid.

#### MISCELLANEOUS ITEMS

Phenols and proteolytic action of pepsin, Crippa (103); insoluble compound of papain and taka-amylase, Akabori & Kasimoto (104); activation of cathepsin by ascorbic acid, inhibition by carotene, Martinson *et al.* (105).

The first stages of casein digestion by trypsin, Grabar (106); electrokinetic potential of protein-pepsin compounds, Loiseleur (107); specificity of cathepsin, Goldstein (108); rôle of cystine in proteolytic digestion, Csonka (109); specificity of acylases, Utzino, Tsunoo, Fujita & Mori (110, 111, 112, 113); property of acylase, Abderhalden & Abderhalden (114).

Action of arginase on proteins and enzymic breakdown products from protein, Kraus-Ragins (115).

#### BIOLOGY

##### PHYSIOLOGY, PATHOLOGY, PHARMACOLOGY

The splitting of carnosine ( $\beta$ -alanylhistidine) by extracts of rat and rabbit livers has been reported by Garkawi (116). According to Weil & Russell (117) the aminopolypeptidase of rat plasma has a pH-optimum of 7.5. Its activity remains constant during tumor or embryonic growth. The plasma proteinase activity, on the other hand, drops considerably under these conditions but returns to the normal level when the tumor is removed by operation, or after termination of pregnancy. Abderhalden & Hanson (118) studied the polypeptidases in blood plasma and found both "prolinase" (Grassmann) and

prolidase (Bergmann). If organs are irradiated with x-rays, proteinases specific for these organs appear in the urine [Abderhalden (119)].

According to Kundu, De & Ghosh (120) the proteinase of venoms from *Echis carinata*, Krait, Cobra, and Russell's viper are essentially of the trypsin type. The blood-clotting principle in venom of *Crotalus t. terrificus* is of proteolytic nature and has albumin-like character [Slota & Fraenkel-Conrat (121)].

#### COMPARATIVE PHYSIOLOGY, EMBRYOLOGY, CYTOLOGY, ETC.

An extensive series of investigations on the enzymes of the intestinal tract in insects [Schlottke (122, 123, 124), Müller (125)] strongly supports the view that the aminopeptidases (including dipeptidases) are typical endo-enzymes (compare also the next section). According to Ågren (private communication) pure pancreatic juice contains traces only of these enzymes. Van Weel & Engel (126), investigating the secretory cycle of pancreas, were able to demonstrate that the activity of the carboxypolypeptidase in the cells of this organ runs parallel with their content of secretion granules. No variation in dipeptidase activity was observed, indicating that the latter enzyme, in contrast to the former, is not secreted. This agrees perfectly well with the observation by Holter (127) and Holter & Kopac (128) that the dipeptidase in certain cells (marine ova and amoebae) is not bound to the granular material. The unquestionable fact that dipeptidase is found wherever growth or any kind of secretional synthesis occurs indicates too that the function of this enzyme is within the cell [compare Holter (129)]. These considerations emphasize the necessity of reconsidering the question of the participation of aminopeptidases in the external breakdown of food in the duodenal and lower part of the intestinal tract of higher animals as well. It must be remembered that the secretion of the pylorus (130, 131) was found to be free of enzymes and that possibly the content of aminopeptidases in the lumen of duodenum may originate from "unphysiologically" extricated and autolyzed mucous cells.

In a short notice, Levy & Palmer (132) described the dipeptidase distribution in the cephalic region of the chick embryo. Goldstein & Ginzburg demonstrated cathepsin in the yolk sack, but not in the yolk itself nor in the amniotic or allantoic liquids (133). Galvyalo & Goryukhina (134) observed an increase of proteolytic enzymes in the stomach during development.

Doyle (135) has shown that in eggs of *Psammechinus miliaris* no change in dipeptidase activity occurred upon fertilization and during the first stages of development. Holter, Linderstrøm-Lang & Lanz (136) were unable to demonstrate any change in dipeptidase activity in relation to early differentiation in eggs of the same sea urchin.

#### BACTERIOLOGY

The chemistry of bacterial proteases has interested several investigators during recent years [compare Virtanen, *et al.* (137, 138, 139); Maschmann (140, 141); and several others]. Special attention has been directed toward the bacteria which liquefy gelatine and a series of papers has appeared demonstrating with certainty that these bacteria secrete at least one proteinase into the surrounding culture medium (137, 142). The nature of this proteinase is not yet settled. According to Maschmann (143, 144, 145, 146, 147) the proteinases of anaërobic pathogenic bacteria are of two kinds: one, a "collagenase" which is secreted and liquefies gelatine without activation by cystein; the other, the "anaërobiase," which acts on clupein only after activation by cystein. The latter, probably an endo-enzyme, is not secreted by the bacteria but appears in the culture medium due to autolysis. Specific differences between the enzymes in different bacteria have been found: the collagenase in *Bacterium perfringens* splits only gelatine; that in *Bacterium botulinus* splits ovalbumin and casein as well (141); in *Bacillus histolyticus* the action of the collagenase (or perhaps a third enzyme) toward casein is inhibited by serum (144); in *Bacillus sarcophysematos* no anaërobiase is present in the culture medium (145); etc., etc. The enzymes seem to be very poorly defined and their action seems to depend considerably on method of preparation, etc. According to Kocholaty, Weil & Smith (142) there is no reason even to distinguish between two proteinases in the culture medium of *B. histolyticus*. They find the same percentage activation by cystein-Fe<sup>++</sup> when gelatine and when clupein are used as substrate.

Kocholaty & Weil (148) have further described an interesting case of adaptation in *B. histolyticus*. It appears that the specificity of the proteinase is determined by the protein (gelatine or casein) present in the culture medium, *viz.*, the proteinase secreted by bacteria grown on casein substrate is able to digest casein but not gelatine and *vice versa*. Also adaptation to different acidities in culture media (adaptive change of pH-optimum) seems to occur.

As regards peptidases all investigators seem to agree that they are endo-enzymes and only appear in small quantities in the culture medium. Kocholaty, Smith & Weil (149) disrupted the bacterial cells by use of a supersonic oscillator and determined the peptidases liberated. The polypeptidase of *B. histolyticus* could be activated by magnesium ions (substrate: leucylglycylglycine).

Berger, Johnson & Peterson (150, 81) have investigated the peptidases of *Leuconostoc mesenteroides*. They report the presence of at least two dipeptidases and three polypeptidases.

Imaizumi and Utzino, Imaizumi & Nakayama (151, 152, 153, 154) have studied the proteinases, peptidases, acylases and amidases from a series of bacteria. Nonliquefying bacteria do not secrete proteinases. In *B. subtilis*, *B. proteus*, *B. prodigiosus*, *B. pyocyaneus* and *B. coli* urease is present along with other amidases which split acetamide, benzamide, oxamide, etc. Acylases or carboxypolypeptidases are present in *Staphylococcus aureus*, *B. prodigiosus* and other species.

#### MISCELLANEOUS ITEMS

Stability of *Abwehr*-proteinases in dried serum, Abderhalden (155); proteolytic breakdown of antidiphtheria serum, Modern & Ruff (156); destruction of tetanus toxin by trypsin after treatment with papain, Ray (157); influence of benzopyren on autolysis, Rondoni & Beltrami (158); luteinizing activity of pituitary extracts destroyed by trypsin, McShan & Meyer (159); activation in tumors, Eisen & Langer (160); proteolytic activity of polymorphonuclear leucocytes, monocytes and epitheloid cells, Weiss, Kaplan & Larson (161).

Proteolytic enzymes in sprouted wheat, Mounfield (162); papain-like enzyme in seeds of *Butea frondosa*, Chatterjee, Ghosh & Chopra (163); proteolytic enzymes in *Cribrina artemisia*, Takemura (164); trypsin-like enzyme in *Anemonia sulcata*, Saviano (165); strong activity of cathepsin in leg of tadpole (*Pelobates fuscus*) during the first stage of development, Goldstein & Zipero-witsch (166); cathepsin in protozoa (*Glaucoma piriformis*), Lawrie (167); asparaginase in bacteria, Utzino & Imaizumi (168).

#### ANALYTICAL

Anson (169) has described in detail his hemoglobin method for the estimation of proteolytic enzymes. Greenstein (170) has proposed the use of cystinyl peptides as substrates for estimations of aminopolypeptidase and dipeptidase. However, before changing to such substrates, it may be interesting to take up again the investigation of the individuality of these enzymes. Popovici & Radulescu (171) have proposed to use dioxane instead of alcohol or acetone in Harris' method for the determination of amino and carboxyl groups. [That



Harris' method of amino group estimation is in principle different from the one described by Linderstrøm-Lang is not generally understood—compare the recent mistake by Calvery (172)]. Toennies & Callan (173) have studied the perchloric-acetic acid method of amino acid titration. An important new method for gasometric determination of carboxyl groups, based on the reaction of amino acids with ninhydrine, has been described by Van Slyke & Dillon (174, 175, 176) [compare (177)]. Linderstrøm-Lang & Lanz (178) have described in detail a dilatometric micromethod for the determination of peptidase activity.

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## NONPROTEOLYTIC ENZYMES

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There are as yet few examples of nonproteolytic enzymes that have been isolated in crystalline form. In spite of this it has been possible in several instances to demonstrate that enzymes are complexes of protein carriers (apoenzymes, "*Träger*") of high molecular weight with dialyzable prosthetic groups (coenzymes). In some cases the nature of the active group has been made clear and in some instances the protein carrier has been crystallized (1). It must be pointed out that for substances with very high molecular weights, such as protein carriers of enzymes, mere crystallization is not an infallible criterion of purity and homogeneity. To determine whether such crystalline substances are pure and homogeneous special methods must be resorted to. Some of these methods, such as ultracentrifugal sedimentation and electrophoretic measurements, are assumed to give reliable results as to the homogeneity of substances of high molecular weight (2).

All available evidence seems to indicate that the actual reaction within the substrate molecule is brought about by the prosthetic group of the enzyme, whereas the absolute velocity (the enzymic activity) and the specificity are mostly determined by the carrier. It must be kept in mind that in many cases no actual evidence exists for the possibility of a prosthetic group being reversibly split off from the enzyme molecule. Naturally we have to believe that some sort of active group occurs in every enzyme, but nothing prevents us from assuming that these groups may, for instance, reside in the amino-acids of polypeptide chains. Even if a detachable prosthetic group does exist in certain cases, the protein carrier as well must possess active groups binding the substrate and determining the specificity. The formation of the apoenzyme-coenzyme complex (holoenzyme) also requires the existence of specific groups in the carrier. The carrier of an enzyme is as specific as the so-called specific group. Under no circumstances can it be assumed that every protein can act as an enzyme carrier if, for instance, it possesses merely a suitable degree of dispersity. The same coenzyme may combine with different carriers, thus forming enzymes catalyzing the same type of reaction but having a different specificity. The cozymase, for example,

combines with various apoenzymes to form dehydrogenases of different substrate specificity. An iron-porphyrine compound in combination with some carriers acts as a catalase and with others as a peroxidase, etc. The different phosphatases, distinguishable through their activity-pH-curves, may have the same active group but may be carriers of different electrochemical properties.

One of the fundamental notions of enzyme chemistry is that of the enzyme-substrate complex as the reacting molecule. The existence of such complexes has been directly demonstrated in some cases. Medwedew (3), on the other hand, denies the significance of the enzyme-substrate complex, although he admits its existence. In his opinion, enzyme action consists essentially of "collisions of higher order" between activated enzyme and substrate molecules. The new theory cannot explain much more than the old and besides such a mechanism is not convincingly demonstrated by the experimental material brought forth by Medwedew as, for instance, in the case of carboxylase. Accepting the conception of the enzyme-substrate complex, Taylor (4) has introduced the concept of electronic induction, as the primary cause of enzyme action. The electronic strain conveyed along the substrate molecule from the valency bonds formed between enzyme and substrate is the source of the action. As to the question of "Lyo- and Desmoenzymes," cf. Bamann & Salzer (5).

From the observation that, in cells, some enzymes are increased in quantity by the presence of the corresponding substrate whereas others seem to be unaffected under the same conditions, enzymes have been classified either as "adaptive" or as "constitutive" enzymes [Karström (6)]. Quastel (7) found this classification inadequate to account for certain results with *M. lysodeicticus*. The rational explanation appears to come from considering enzymes as cell metabolites following the same laws as other metabolites.

Interesting reviews on the use of isotopic indicators in biological research have been published by Hevesy (8) and Krogh (9) [formation of organic constituents of the body, questions of permeability, (10), etc.]. The methods may be applicable in many instances. As to the use of radioactive phosphorus refer to the section—Phosphate-transferring Compounds in Fermentation and Glycolysis (page 64). Interesting work on radioactive phosphorus as an indicator of phospholipid metabolism has been carried out by Chaikoff *et al.* (11).

## ESTERASES

Langenbeck in 1934 reported that benzoylcarbinol and some other substances behave as esterase models. Their action was claimed to consist of two phases: (a) Transesterification between ester and active alcohol, and (b) hydrolysis of the new ester by water. Ionescu & Cotoni (12) were, however, unable to demonstrate any action of benzoylcarbinol. The specificity of pancreatic lipase was studied in several papers by Balls *et al.* A maximum rate of hydrolysis of straight chain glycerides was observed when the constituent acids had a chain length of from seven to ten carbon atoms. Olein, on the other hand, behaves as if it had a chain length of nine instead of eighteen carbon atoms. The rate of hydrolysis of fats of higher acids is much more affected by temperature than is that of esters of lower acids. Tristearate is not appreciably affected below 30° C but is strongly attacked between 40° to 50° C (13). The ethyl esters are split almost as readily as the glycerides and the rate varies in the same way with the chain length of the acids. Only primary ester groups seem to be attacked. In fats, however, the secondary ester groups are hydrolyzed; therefore, it must be concluded either that this group has other properties than that of simple secondary esters or that migration of  $\beta$ -to  $\alpha$ -position takes place (14). Pancreatic lipase readily hydrolyzes benzylbutyrate and stearate, the latter only at higher temperatures. The butyrate is also split by liver enzyme (15). Hartwell (16) studied the hydrolysis of different fats by pancreas lipase: coconut oil is split more readily than all other fats; palm-kernel and castor oil more rapidly than butter fat; other oils and fats at approximately the same rate but more slowly than butter fat. The retarding action of sodium salts of fatty acids has a maximum at a chain length of from twelve to thirteen carbon atoms (Holwerda, 17) and is neutralized by sodium-glycocholate, which is supposed to cause a better adsorption of enzyme to fat surface. Chrzaszcz & Janicki (18) determined the lipolytic activity of the pancreas of animals of different age. The enzymatic synthesis of esters was studied by Sym (19) who used acetone-dried preparations from pancreas in different organic solvents. The synthesis of the butyl esters of several acids was demonstrated and the influence of the nature of solvent studied. The synthetic action of organs and tissues from different animals was compared by Cedrangolo (20). Esterase content in normal and pathological urine was determined in 650 cases by Zorn (21). The value of the esterase determination for diagnosis and prognosis was discussed (review of



literature). Kraut & Burger (22) found that horse serum hydrolyzes the acetone-soluble fraction of tubercle-bacillus wax slowly, whilst human serum has no activity. There is no correlation between lipase content of serum and occurrence and course of tuberculosis. Tubercle-bacillus fat is hydrolyzed by pancreas lipase from pig but only very slowly by liver extracts from this animal. Virtanen & Roine (23) found that an artificial increase in the lipase content of serum has no action on rabbit tuberculosis.

Choline esterase has been the subject of several investigations of which two may be mentioned: Glick (24) studied the specificity of this enzyme; Clark and coworkers (25) studied the kinetics of its action. The hydrolysis of cholesterol esters by cholesterae has been treated by Nedswedski (26), by Klein (27) and others. In connection with work on hydrolysis and synthesis of fats and esters some investigations on the degradation of fatty acids in the body are of interest: Toennisen & Brinkmann (28), Blixenkrone-Möller (29), Stark & Cohen (30).

#### PHOSPHATASE

Work on the occurrence of phosphatases and phosphoric esters and their significance in biological processes has advanced in many directions. Bamann & Meisenheimer (31) made the interesting observation that certain metal hydroxides, as for instance lanthanum hydroxide, act as "inorganic phosphatases" in a faintly alkaline medium. Albers and coworkers showed that phosphatase is a complex of a cophosphatase and an apophosphatase, forming together the holophosphatase (32). At pH 5 to 6 the kidney phosphatase dissociated and the cophosphatase could be separated from the apoenzyme by dialysis. The nondialyzable, inactive apoferment was reactivated by the cophosphatase. The complex nature of top yeast phosphatase which has optimum activity at pH 4 was also demonstrated. As all phosphatases have the same action on their substrates, it is probable that they have the same active group (cophosphatase). In fact, the authors succeeded in activating coenzyme-free kidney phosphatase at pH 9 by top yeast phosphatase, which itself is quite inert at this pH. In a corresponding way the top yeast phosphatase was "synthesized."

One of the most intricate questions in this field concerns the specificity and classification of phosphatases. Bamann & Gall (33) pointed out that in many cases the reported absolute specificity may be only relative. Nevertheless they were able to confirm the results of

Bauer (34) and others that pyrophosphatases have their own specificity. The "isodynamic" phosphatases may be differentiated by their pH-optima. In a study of bone and snake-venom phosphatases Gulland & Jackson (35) found that mono- and diesterases exist as mixtures. The metatarsal bones from sheep forelegs are a cheap and suitable material for large scale preparations of the phosphatases. Both phosphatases are adsorbed on charcoal. The observation that mono-esterase but not diesterase was eluted by borate buffer at pH 8.6 was utilized for the separation of the enzymes. Seven snake venoms contained both esterases, five only diesterase. The snake family is not the deciding factor. Reis (36) revealed the existence in many tissues of a 5-nucleotidase specific for t-adenylic acid and inosinic acid. The enzyme also occurs in some snake venoms. Gulland & Jackson (37) confirmed that the enzyme acts on adenosine- and inosine-5-phosphoric acid but not on adenosine-3-phosphoric acid,  $\alpha$ - and  $\beta$ -glycerophosphate or monophenyl phosphate. In this connection it is interesting that adenosine and phosphoric acid in the presence of yeast give t-adenylic acid [Ostern *et al.* (38)]. Guanosine and d-ribose are not esterified. Yeast adenylic acid can be enzymatically transformed to t-adenylic acid.

Continuing earlier work Pett & Wynne (39) studied some bacterial phosphatases. Marked differences exist between two strains of *B. subtilis*. It is to be expected that differences of this kind may be valuable for the differentiation of bacterial species. It is very interesting that *Alcaligenes faecalis*, which does not produce acids or gas and probably has no glycolytic system, contains an active phosphatase.

Several papers have dealt with the activation of phosphatases with magnesium ions; Bamann & Salzer (40), Cedrangolo (41), Cattaneo *et al.* (42), Giri (43). In contrast to certain earlier investigations King & Delory (44) found no action of ascorbic acid on the hydrolysis of common esters. Freeman *et al.* (45) determined the phosphatase activity of serum after several kinds of hepatic injury (rise of activity). Anderson & Squires (46) found a distinct elevation of phosphatase activity in dog serum in phlorhizin diabetes. Thannhauser *et al.* (47) suggested that the high activity of pathological sera is due to activation rather than to increased amount of enzyme [cf. Freeman & Chen (48)]. Waldschmidt-Leitz, Samec & Mager (49) demonstrated that amylophosphatase from barley or malt, which has a strong action on potato starch, does not liberate phosphorus from wheat starch, although both starches have about the same phos-

phorus content. This may be explained on the assumption that potato starch is a normal ester of phosphoric acid whilst the phosphorus in wheat starch is also bound to nitrogenous substances, possibly phosphatides [Posternac (50)]. Kidney phosphatase, having a relatively small action on potato starch, rapidly dephosphorylates wheat starch. This phosphatase also acts on creatine phosphoric acid which is not attacked by amylophosphatase. Further work on mono- and diesterases has been reported by Roche & Latreille (51, 52). Neuberg & Fischer (53) made the interesting observation that inorganic triphosphoric acid is split into orthophosphoric acid by enzymes from *Aspergillus oryzae* and yeast.

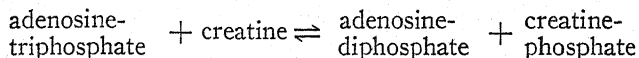
An enzyme [nuclease, Jones (54)] capable of decomposing yeast nucleic acid into acid-soluble products was prepared by Dubos & Thompson (55) from pancreas and many other tissues. No inorganic phosphate is liberated. Thymonucleic acid is not attacked, but pneumococci are changed from the Gram-positive to the Gram-negative state. The enzyme is a protein, rapidly destructible by crystalline pepsin but resistant to trypsin and chymotrypsin. It is of particular interest that the enzyme is quite stable at 95° C. The decomposition rate of nucleic acid rises with temperature up to 70° C, then decreases to zero at 85° C. This inhibiting effect of high temperatures is reversible. Schmidt & Levene (56) confirmed these results. Concerning sulfatase cf. (58).

#### PHOSPHATE-TRANSFERRING COMPOUNDS IN FERMENTATION AND GLYCOLYSIS

Phosphoric esters play a very important part in fermentation and glycolysis. Adenylic acid and allied substances act as coenzymes in the transfer of phosphoric acid to carbohydrate or its conversion products ("*Phosphatübertragung*"). To the known adenosine nucleotides, adenylic acid, adenosinetriphosphoric acid, adenosinediphosphoric acid, diadenosinepentaphosphoric acid (heart nucleotide), Kiesel & Meyerhof (59) have added the diadenosinetetraphosphoric acid as well as the corresponding pyrophosphate complex. All these substances seem to act as phosphate carriers [Ohlmeyer & Ochoa (60)]. The same action is produced by alkali-inactivated cozymase (Cz), but not by active cozymase or by cozymase inactivated by strong acid [Euler & Adler (61), Bauer (62), Vestin (63)].

The first stage in glycolysis and fermentation consists in the

phosphorylation of fermentable hexoses or glycogen (starch), the adenylic acid system being essential. From the polysaccharides the Cori-ester (glucose-1-phosphoric acid, phosphate- $\alpha$ -glucoside) is formed (64), but it is rapidly converted into the 6-ester. The corresponding  $\beta$ -glucoside is not attacked. The 6-glucose ester partly yields 6-fructose ester, and the next phase is considered to be the phosphorylation of the latter to fructose-1, 6-diphosphate (Harden and Young's ester). Muscle glycolysis differs from fermentation inasmuch as creatine and its phosphoric esters play a rôle as phosphate carriers in the muscle system. Creatine is phosphorylated partly by phosphopyruvic acid and partly by inorganic phosphate in a reaction coupled with oxido-reduction. Adenosinetriphosphate is essential in this case. The reaction



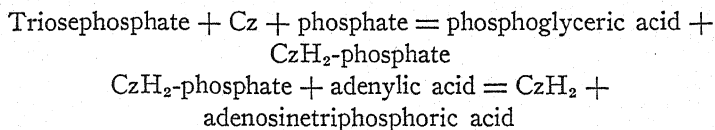
is quite reversible. In yeast and in most animal tissues hexosediphosphate is changed to a mixture of dihydroxyacetone phosphate and glyceraldehyde phosphate (aldolase, Lohmann). Muscle extract converts each of these substances into a mixture of both of them. The glyceraldehyde phosphate is, in a coupled reaction, oxidized to 3-phosphoglyceric acid, which is converted to the corresponding 2-acid (yeast, tissues). The enolase (Lohmann) changes this acid into phosphopyruvic acid which is dephosphorylated in presence of phosphate carriers. A review of the present knowledge of phosphorylation is given by Parnas (65), including the use of radioactive ("labelled") phosphorus,  $P^{32}$ , in the chemistry of physiologically active phosphorus compounds. The phosphorylation of carbohydrates and their conversion products is brought about in two different ways:

a) *Phosphorolysis* (disruptive phosphorylation), enzymatic cleavage of polysaccharides under the uptake of phosphorus: Glycogen + phosphate = hexosemonophosphate. Experiments with radioactive ( $P^{32}$ ) disodium hydrogen phosphate show that inorganic phosphorus really enters into the reaction: The resulting Embden-ester has the same radioactivity as the inorganic phosphate used. The primary product is the ester discovered by Cori & Cori in 1936 (see above). The transfer of the phosphoric acid group from the first to the sixth carbon atom is caused by the enzyme phosphoglucomutase [Cori *et al.* (66)]. The name is given in analogy with phosphoglyceromutase

which transfers phosphoric acid from the 3- to the 2-position in phosphoglyceric acid (Meyerhof & Kiessling 1935). Synthetic mannose- and galactose-1-phosphate are not changed by the enzyme [Colowick (67)]. Schäffner & Specht (68) demonstrated that glycogen is phosphorylated by yeast in the absence of Cz, adenosinetriphosphate, hexosediphosphate and magnesium ions. The hexosephosphatase and the dehydrogenases play no part in this reaction. The glycogen-phosphatase is much more stable than hexosephosphatase and is not identical with phosphorylase. Cori and coworkers state that the synthesis of Cori-ester from glycogen in rabbit tissue extracts is greatly accelerated by adenylic acid and to a minor degree by inosinic acid (69). When pyrophosphatase is present adenosinetriphosphate, which alone has only a weak action, produces about the same activation as adenylic acid. Kendal & Stickland (70) reported that for the reaction — carbohydrate  $\rightarrow$  Cori-ester — adenylic acid or adenosinetriphosphoric acid is essential whereas inosinic acid is only slightly active. These authors found that the reaction is strongly stimulated by magnesium ions [cf. Cori *et al.*, (71)]. The second phase—Cori-ester  $\rightarrow$  glucose-6-phosphate—is greatly activated by magnesium, manganese or cobalt ions (66). The third phase—glucoseester  $\rightarrow$  fructoseester—is probably not influenced by magnesium ions.

b) *Phosphorylation coupled with oxido-reduction.*—Hydroxy groups of sugars or their conversion products are esterified with phosphoric acid arising from the above-mentioned "phosphate donators". Coupled with this reaction is a chain of processes, in which Cz is hydrogenated. According to Meyerhof (72) this reaction is to be interpreted as the coupling between two reactions, one consisting in the hydration of Cz by triosephosphate and the other in the esterification of the adenylic acid system. For each molecule of Cz hydrogenated, one molecule of inorganic phosphate is esterified until equilibrium is reached. The oxidation of  $\text{CzH}_2$  by acetaldehyde or pyruvic acid and the hydrogenation of Cz by alcohol or lactic acid is not connected with any uptake of phosphate. The reaction involving the hydrogenation of Cz and the uptake of phosphorus is quite reversible. In the presence of small amounts of Cz, phosphoglyceric acid is reduced to triosephosphate if suitable phosphorus donators such as phosphopyruvic acid or creatinephosphate are present. The reversed reaction can also be demonstrated: Cz is oxidized by phosphoglyceric acid and one molecule of phosphoric acid is set free from adenosinetriphosphoric acid. The oxidation of triosephosphate during hydrogenation of Cz yields

energy for the esterification of adenosinediphosphoric acid. Needham (73) suggested that reduction and phosphorylation take place in the same molecule:



The phosphorylation in kidney tissue disappears in the absence of oxygen [Kalckar (74)] but reappears on supplying oxygen. This is due to a coupled reaction which in this case manifests itself in the proportionality between phosphate and oxygen uptake. Hitchings *et al.* maintained (75) that differences exist in the mechanism of tumor and muscle glycolysis in so far as the products formed from glycogen in the presence of sodium fluoride are found to be different in these two systems, and furthermore since tumor extracts fail to catalyze the reaction between triosephosphate and pyruvate. Boyland & Boyland (76), however, present experimental evidence for the view that in both instances the intermediate processes are identical. Deuticke *et al.* (77) found that in minced liver no formation of phosphoglyceric acid and glycerophosphoric acid take place in the presence of sodium fluoride. This condition is in contrast to that previously noticed in muscle. The normal, unpoisoned liver dephosphorylates phosphoglyceric acid with the formation of pyruvic acid. Liver contains donators which transform phosphoglyceric acid and pyruvic acid into lactic acid. Phosphoglyceric acid is an intermediate product in liver glycolysis. When glycerophosphoric acid is present the formation of lactic acid from phosphoglyceric acid is not increased. Part of the pyruvic acid is lost without the formation of lactic acid. Pyruvic acid, and not lactic acid, has a central position in the carbohydrate metabolism of liver. Heart muscle transforms hexosediphosphate in presence of sodium fluoride into laevorotatory phosphoglyceric acid which is dephosphorylated in the unpoisoned muscle.

#### CARBOHYDRASES

*α-Glucosidase*.—Weidenhagen's "general specificity theory of glucosidases" does not seem to be as general as supposed. In confirmation of earlier work [Karström (79), Myrbäck (80)] Leibowitz & Hestrin (81) found that the hydrolysis of saccharose, maltose and *α*-methyl-

glucoside by takadiastase preparations is not caused by one and the same enzyme.

*$\beta$ -Glucosidase.*—Much interesting work has been carried out on this enzyme particularly by Helferich and by Veibel. It has been established that the same enzyme splits  $\beta$ -*d*-glucosides and  $\beta$ -*d*-galactosides [Helferich (82)]. In work on the hydrolysis of triethylcarbinol- $\beta$ -*d*-glucoside Veibel & Lillelund made observations on the significance of steric hindrance for enzymatic hydrolysis of glucosides (83), and have given directions for the standardization of  $\beta$ -glucosidase preparations (84). Helferich *et al.* (85) determined the hydrolysis of the 6-halohydrins of  $\beta$ -glucosides. In the case of vanillin- $\beta$ -*d*-glucosides the rate of hydrolysis decreases with the "volume" of the halogen substituent (Cl, Br, I and F). In studies on the hydrolysis of mono- and bisglucosides of alcohols and phenols with two hydroxyl groups, they found (86, 87) that the hydrolysis rate of the bisglucoside of catechol is only about a tenth of that of the monoglucoside. When the 6-hydroxyl of one of the sugar residues is esterified with methyl sulfonic acid, this glucosidic group is no more attacked by the enzyme but the other glucose residue is split off at about the same high rate as is the glucose residue in the monoglucoside. These results can be interpreted on the assumption (86, 88) that the enzyme combines with the sugar residue of the glucoside. It is suggested that the pyranose ring in the sugar is indispensable, but not the  $\text{CH}_2\text{OH}$ -group, nor is the nature of the aglucone the deciding factor. Possibly a carbohydrate or a similar grouping in the enzyme is responsible for the attachment of the enzyme. A summary of work on  $\beta$ -glucosidase is given by Helferich (89).

*Saccharase (invertase).*—Several papers by Oparin, Kurssanow *et al.* are concerned with the reciprocity of hydrolyzing and synthesizing activity of saccharase in the living plant. While in extracts only the former action can be observed, the synthetic action, which was studied by the vacuum infiltration method, is very strong within the cell. It was supposed that the direction of the enzyme action is governed by the degree of fixation of the enzyme in the cell (90, 91).

*Amylases.*—Work by Blom *et al.* (92) definitely shows that the  $\alpha$ -amylases are not identical with the  $\alpha$ -glucosidases. This is also most probably the case with  $\beta$ -amylases and  $\beta$ -glucosidases. By repetition of earlier investigations it has been definitely established that an amylase is present in muscle extracts [Willstätter (93), Lehmann (94), Mystkowski (95), Walker (96)]. Its amount is small and it probably

plays no part in the glycogenolytic cycle, phosphorolysis and amylolysis being two distinct processes.

Several papers have dealt with the nature of the inactive amylase in barley and similar material. Great variations seem to exist in barley samples grown under different conditions [cf. (97) and (98)]. A very interesting study on the distribution of amylase in the outer parts of barley seed was published by Linderström-Lang & Engel [(99) cf. (100)]. Papers by Ohlson & Thörn (101) and by Giri & Sreenivasan (102) deal with the fate of amylases during ripening and germination of barley and rice. The extremely important and intricate question as to the number of enzymes in naturally occurring materials was studied by Blom *et al.* (103) who found that pancreas amylase as well as the bacterial enzyme superclastase are pure  $\alpha$ -amylases. The reaction products of such enzymes were also investigated (104). Holmbergh (105) reported on the inactivation of solutions of malt amylase caused by shaking. He also described (106) a convenient method for preparing large amounts of relatively pure malt amylase ( $\alpha$ - +  $\beta$ -amylases). [On the action of wheat amylases, cf. (107), amylase of human liver (108), action of heavy water (109).]

The reviewer has been engaged in work on the starch-splitting enzymes and particularly on the nature of their reaction products, i.e., beside fermentable sugars (maltose and glucose) the so-called stable dextrins. In contradiction to certain earlier investigations (cf. Pringsheim: *Die Polysaccharide*) it was proved that these dextrins as well as maltose must be considered primary products, i.e., parts of starch molecules which for some reason or other are not, or only extremely slowly, attacked by normal amylases (110, 111). It must be emphasized that theories on the constitution of starch must not only explain the chemical behavior of starch but also, in an acceptable way, the action of the starch-splitting enzymes and the nature of their reaction products. The investigations of the last few years have shown beyond a doubt that the sundry amylases belong to clearly different groups, not only because some amylases yield  $\alpha$ -maltose and others  $\beta$ -maltose, but also because the various amylases produce, besides maltose, stable dextrins which vary in nature. It must be concluded that the different amylases attack only or preferably some special linkages in the starch molecules (112). It seems impossible to assume that the distance of a glucosidic linkage from the reducing or nonreducing end of the chain could alone determine whether or not the linkage will be attacked by a certain amylase (113). The conclusion remains to be drawn that the



starch molecules cannot be as simple as postulated by the formula of Haworth (149). In order to explain the action of different amylases on starch and the formation of the various stable dextrins the writer has put forward the following hypothesis:

a) Maltose, the main product of enzymic starch degradation and of the breakdown of starch by certain nonenzymatic agents, as for instance acetyl bromide (Karrer), is formed from such parts of the starch molecules as are built in accordance with Haworth's formula.

b) The formation of various stable dextrins indicates the existence in the starch molecules of parts having a constitution different from that postulated by Haworth such as: linkages between the glucose residues other than the  $\alpha$ -1, 4-linkage; other positions of the oxygen bridge in certain glucose residues; substitution of certain hydroxyls by phosphoric acid or other groupings; etc. The starch molecules have for the most part the constitution illustrated by Haworth's formula, but at intervals in the maltose chains anomalies occur.

c) Possibly the normal amylases are unable to attack chains shorter than a certain minimum length (hexasaccharides for instance). It might be that some stable dextrins are formed because of this condition. However, the formation of *all* stable dextrins cannot be accounted for in this way (114).

Starches of different origin have been hydrolyzed as completely as possible by different amylases and the stable dextrins after removal of the sugars by fermentation have been precipitated with alcohol, purified, and as far as possible isolated in fractions of different chain lengths. All stable dextrins have proved to be mixtures of a great number of saccharides of different molecular weight. In some cases, in spite of a very thorough degradation, the stable dextrins contain from three (or possibly two) to about twenty glucose residues. There is in this respect a marked difference between starches rich in phosphorus, as potato or wheat starch, and corn or rice starch which contain little phosphorus. Among the taka-dextrins from corn starch (115), for example, one fraction apparently containing hexasaccharides is very large, whereas dextrins of a higher molecular weight are almost absent. All stable dextrins have a reducing power roughly corresponding to one aldehyde group per molecule. (Molecular weight from diffusion data!) Anhydrides and rings are thus not present. Almost all dextrin fractions contain more or less phosphorus. In most cases the phosphorus of starch accumulates in the dextrin fractions of highest molecular weight.

The  $\beta$ -amylase from ungerminated barley (saccharogenic amylase) yields 60 per cent of maltose and about 40 per cent of starch-like dextrins giving a blue color with iodine. The enzyme action starts from the nonreducing ends of the chains, and maltose molecules are split off until an anomaly in the constitution puts a stop to the enzyme action. A limit-dextrin of high molecular weight is left over (111). After heating starch with dilute acids products of a low molecular weight (1000 to 2000) were isolated. When these were acted upon by  $\beta$ -amylase 30 to 60 per cent of maltose was formed (113, 116, 117). The fact that some fractions of the dextrins formed have a molecular weight as high as that of the substrate indicates that the enzyme acts to a different degree on different molecules. The cessation of the enzyme action is not due to the shortening of the chain length but to the existence of constitutional anomalies. These bear no relation to the high molecular weight of the natural substrate, nor to any aggregation of the true molecules of starch.

The  $\alpha$ -amylases break the molecules far from the ends of the chain. [All amylases are independent of the existence of free reducing groups in the substrates, since starch and dextrins when oxidized with hypoiodite remain unaltered in their behavior towards the amylases (116).] The primary action of these enzymes ("dextrinogenic amylases") consists in the formation of dextrins yielding no color with iodine and having, in the case of potato starch, a molecular weight of about 7000. The enzymes act not only on the natural substrates of large molecular weight but on products with molecular weights of 1000 to 2000 as well (113); the conclusion drawn is that the action is unrelated to chain length or distance of the glucosidic linkage from chain ends. It must be inferred that the linkages primarily attacked by  $\alpha$ -amylases are different from the other glucosidic linkages (maltose linkages).  $\alpha$ -Amylases and natural mixtures of  $\alpha$ - and  $\beta$ -enzymes finally yield fermentable sugars and dextrins of low molecular weight, mostly from 500 to 2000. Considerable quantities of trisaccharides were isolated in several cases. Some of the dextrin fractions on treatment with acetylbromide formed no acetobromomaltose (119) whereas check experiments with starch and maltose gave the yields found by Karrer (150). This suggests that these dextrins are not exclusively built up according to the Haworth formula. It is as yet uncertain whether disaccharides other than maltose are present among the breakdown products of starch.

## UREASE

Sumner, Gralén & Erikson-Quensel (120) investigated the behavior of solutions of crystallized urease in the ultracentrifuge. The sedimentation curves revealed the presence of but traces of foreign material and the diffusion curves were very close to the ideal distribution curves. The crystalline urease is obviously a homogeneous protein. Its partial specific volume is 0.73. From the sedimentation data a molecular weight of 483,000 was calculated. In view of the existence of sulfhydryl groups in the urease (Sumner & Poland 1935) and their possible significance for the enzymatic activity, an investigation by Pillemer *et al.* (121) of the effect of irradiation and oxidation on the crystalline enzyme is of great interest. Urease solutions inactivated under controlled conditions through aëration or by iodine could be reactivated by reducing agents such as potassium cyanide or hydrogen sulfide whereas the inactivation caused by ultraviolet light was quite irreversible. Tissue extracts reactivated the oxidized but not the irradiated enzyme. The reversibly oxidized enzyme induced in animals the formation of true antiurease as does the active urease. A rabbit immunized with the oxidized enzyme could withstand ten lethal doses of the active enzyme. The irradiated enzyme produced no antibody. Irradiation probably causes denaturation of the protein molecule whereas in the controlled oxidation only some side chain or chemical linkage in the compound is attacked. It can be reasonably assumed that the sulfhydryl groups are oxidized to dithio groups. Cross-precipitation experiments with antisera from active and inactivated enzyme disclosed the possibility that the SH-group is the carrier of the immunological specificity of the compound.

The question of the tryptic action on crystalline urease, which has been a matter of dispute for some time, has been settled: The formation from urease of products with lower molecular weight is accompanied with a parallel decrease in activity, i.e., the activity is bound to the protein itself. The absorption spectrum of urease does not alter in presence of urea (122).

## ARGINASE

The chemistry of this enzyme is dominated by work on the significance of certain metal ions for the enzymatic activity (Hellermann & Perkins 1935). Hellermann *et al.* (123, 124) determined the pH-curves of jackbean arginase in the presence of different metal ions. Co<sup>++</sup> and Mn<sup>++</sup> exhibited a very strong activating effect. In the case

of cobalt the pH-optimum was shifted from 9 to about 7.7.  $\text{Ni}^{++}$  had little influence, a fact showing the nonidentity of this enzyme with liver arginase. The possibility was discussed that the activation by the metals is related to their ability to form complexes with arginine. Differences in the pH-curves were found with liver arginase too,  $\text{Co}^{++}$  and  $\text{Ni}^{++}$  shifting the optimum to a less alkaline level. The very active manganese compound has an optimum at about pH 10. Edlbacher & Pinösch (125) found that the enzyme is irreversibly inactivated by pepsin. The inactivation caused by hydrochloric acid alone is reversible, and the activity is restored by the addition of metal ions such as  $\text{Mn}^{++}$ ,  $\text{Co}^{++}$  or  $\text{Ni}^{++}$ . They concluded that the enzyme is a complex of a protein carrier and an active group containing manganese. Edlbacher & Baur (126) demonstrated that yeast arginase is inactivated by dialysis at  $0^\circ \text{C}$  and reactivated by metal ions. With liver arginase the dialysis must be continued for weeks. At a  $0.001 M$  concentration manganese, cobalt, nickel, vanadium, and cadmium salts were found to activate to an equal extent, but manganese is capable of restoring the activity even in  $0.000003 M$  solution, a concentration at which the other metals are without action. It was concluded that the enzyme is a protein with manganese as active group.

A liver enzyme, probably arginase, hydrolyzes  $\text{l}(+)\text{-carbamido-arginine}$  but has no action on arginine hydantoin [Hunter (127)]. Concerning the specificity of arginase Felix & Schneider (128) found that the  $\alpha$ -amino group may be substituted by an acid or peptide residue or changed to a hydroxyl without rendering the substances non-hydrolyzable. The  $\alpha$ -amino- $\epsilon$ -guanidocaproic acid is also hydrolyzed, but at pH 7 to 8, where arginine itself is not attacked. The hydrolysis of guanidineacetic acid is probably caused by a different enzyme. Arginase was used by Kraus-Ragins (129) to determine the arginine with free carboxyl groups in proteins.

#### CARBOXYLASE

The cocarboxylase (Auhagen 1931) was identified with the pyrophosphoric ester of vitamin  $\text{B}_1$  (aneurine) by Lohmann & Schuster (151) in 1937. The coenzyme was isolated in a crystalline state and its formula is probably  $\text{C}_{12}\text{H}_{21}\text{O}_8\text{N}_4\text{P}_2\text{Cl}$ . Enzymatic synthesis of cocarboxylase from aneurine was demonstrated by Euler & Vestin (130) in the presence of dried yeast and adenosinetriphosphoric acid or hexose diphosphoric acid, and by Lipschitz, Potter & Elvehjem (131) with washed dried yeast, hexosediphosphate and boiled tissue

extract. Sodium fluoride (0.04 *M*) was found to inhibit the synthesis when pyruvic acid was absent, but when pyruvate was added, the synthesis proceeded rapidly. The reaction involves the esterification of inorganic phosphate and utilizes the energy derived from the oxidation-reduction between triosephosphate and pyruvic acid. Kinnersley & Peters (132) evolved a method for preparing cocarboxylase using fresh baker's yeast. According to Tauber (133) preparations of duodenal enzyme convert vitamin B<sub>1</sub> into cocarboxylase. As the enzyme was found incapable of hydrolyzing the coenzyme it was classified as a phosphatase. Tauber also described (134) a simple method for the direct synthesis of cocarboxylase from aneurine and pyrophosphate. [As to synthesis of aneurine, cf. Hörlein (135).] Cocarboxylase in the presence of aneurine can be determined by a method developed by Roth (136).

The action of cocarboxylase and its apoenzyme in yeast under anaërobic conditions is well known. The fate of pyruvic acid on aëration in the presence of yeast is described in a paper by Smythe (137). (It probably forms fats or fat-like compounds.) The action of the coenzyme in the animal body is also connected with the metabolism of pyruvic acid [Peters (152)]. Decomposition into carbon dioxide and acetaldehyde does not occur in animal tissues except in heart muscle [Simola & Kallio (138)]. Cocarboxylase is concerned instead with the dehydrogenation of pyruvic acid. The mechanism of its action is possibly the same as that of the pyridine coferments (reduction in the thiazol ring). The cocarboxylase content of tissues, especially of brain, is materially reduced in avitaminosis [Ochoa & Peters (139)]. Administration of aneurine leads to immediate accumulation of both aneurine and its pyrophosphoric ester in the liver. Lipmann (140) demonstrated that the ferment in *B. delbrücki* which dehydrogenates pyruvic acid to acetic acid and carbon dioxide, but is devoid of carboxylase action, has cocarboxylase as the prosthetic group.

#### "CARBOLIGASE"

The formation of acetoin and related compounds in fermentation was ascribed by Neuberger to an enzyme called carbolicase. Its existence has been questioned by several investigators. Dirscherl & Schöllig (141) found that acetoin is formed also in the nonenzymatic decarboxylation of pyruvic acid and accordingly denied the existence of carbolicase. Acetoin when formed in the presence of living

yeast or cell-free juice is optically active. This merely shows that the carboxylase-pyruvic acid complex is active. If maceration extract on addition of acetaldehyde yields a little acetoin, this must be formed through the fermentation of carbohydrates in the juice.

#### FERMENTATION

[Some of the questions connected with fermentation have been dealt with in the section on Phosphate-transferring Compounds (page 64). Most of the other questions belong to the chapter on biological oxidation and reduction. Only a few papers will be mentioned here.]

Genevois (142) gave a summary of the work on fermentation of disaccharides. Dickens (143) proposed a very interesting scheme of carbohydrate metabolism: Hexose is phosphorylated, the monophosphate (Robison's ester) is oxidized to phosphohexonate. The is decarboxylated under the formation of phosphopentonic acid, which is again decarboxylated until pyruvate results. When phosphohexonate is oxidized with juice, obtained by Lebedew's method, in the presence of Codehydrase II (Warburg's coferment) one half molecule of oxygen is taken up and a mixture of phosphohexonic and phosphopentonic acids probably is formed. With impure coferment one mol of oxygen is consumed and one mol of carbon dioxide is evolved per mol of hexonate used. Among the products a phosphorylated, monocarboxylic dihydroxy-acid with four carbon atoms occurs, possibly phosphoerythronic acid. Maceration juice oxidizes *d*-ribose-5-phosphoric acid vigorously, *d*-arabinose- and xylose-5-phosphoric acids, however, more slowly. Dialyzed juice is inactive in the absence of coferment II. Intact cells are impermeable to the esters. The rate of fermentation of ribosephosphoric acid by maceration juice is similar to that of glucose. In its fermentation are formed one mol each of carbon dioxide, phosphoric acid and alcohol and also an unknown product. This unknown product does not reduce alkaline-copper solutions, is not precipitated by neutral lead acetate and contains two or more adjacent hydroxyl groups.

#### CATALASE

Beef-liver catalase was isolated in a crystalline form by Sumner & Dounce (153) in 1935 and found to have an activity, "*Kat f.*" = 28,000, which remained constant after several recrystallizations. Amorphous preparations of horse-liver catalase (method of Zeile & Hellström 1930) gave considerably higher values, a fact which is not

due to differences in the procedure of ascertaining the activity but to real differences in the protein carriers of the enzymes. Partly purified preparations from horse liver were studied in the ultracentrifuge by Stern & Wyckoff (144) who found a molecular weight of from 250,000 to 300,000.

The molecular weight of the crystalline enzyme from beef liver as computed from ultracentrifugal data is 248,000 (Sumner & Gralén, 145). Horse-liver preparations having *Kat f.* values from 55,000 to 60,000 were obtained by Agner (146). The partly purified preparations were freed from a component with very high iron content by fractional precipitation with ammonium sulfate. Final purification was made through chromatographic analysis (tricalcium phosphate). The spectrum of the catalase was determined (distinct maxima at 280 and 400 m $\mu$ ). The preparations contained 15.5 per cent nitrogen,  $0.085 \pm 0.005$  per cent iron and 0.02–0.03 per cent copper, and behaved in cataphoretic experiments as a single substance. In the ultracentrifuge, however, the preparations were found to be inhomogeneous. The sedimentation constant of the iron-porphyrin compound was  $11.2 \times 10^{-13}$ , partial specific volume 0.715, diffusion constant  $4.3 \times 10^{-7}$ , corresponding to a molecular weight of 225,000. A second component, amounting to from 15 to 20 per cent of the preparations, had a sedimentation constant of  $12 \times 10^{-13}$ . By fractional precipitation with picric acid two substances were isolated. The first contained 0.1 per cent iron and no copper. It had a sedimentation constant of  $12 \times 10^{-13}$ . The second contained 0.16 per cent copper and very little iron. Its sedimentation constant was  $3.27 \times 10^{-13}$ . These fractions were probably the same as those observed in the sedimentation experiments. In view of the observations of Kubowitz (147) that polyphenol oxidase is a copper protein, and of Keilin & Hartree (148) that the iron of catalase is reduced by hydrogen peroxide to the ferrous state but immediately reoxidized by molecular oxygen, it seems possible that the copper protein in the catalase preparations may be necessary for the reoxidation of the ferrous iron. The two fractions had a much lower activity than the unfractionated preparation but the activity was not restored by simply mixing the two fractions.

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# POLYSACCHARIDES AND LIGNIN

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## POLYSACCHARIDES

### GENERAL

Cellulose was long considered to be the typical compound of the whole polysaccharide group. Its structure was conceived of as a long homogeneous chain. Now it has been shown that cellulose is a limiting case and almost all of the other polysaccharides do not have simple chain structures. Most polysaccharides consist of intricately branched chains. Following this idea, which has been applied for example to the structure of starch, Schlubach & König (1) developed and experimentally proved such a structure for the fructose anhydrides. There remains yet a question of secondary importance, *i.e.*, whether the branching proceeds from rings or from open chains in the polysaccharide. The two possibilities are shown in Figures 1 and 2 in which every line represents a monose unit. When a polyhexose is methylated and hydrolyzed every nonaldehydic end member (Figure 1 contains three; Figure 2 contains five) yields tetramethylhexose; every branching position yields dimethylhexose (three in Figure 1; four in Figure 2); all remaining members (six in Figure 1 and eleven in Figure 2) produce trimethylhexose. Besides the search for end groups (tetramethylhexoses or trimethylpentoses), which was first undertaken by Irvine & Hirst (2), the tracing of branching positions (dimethylhexoses or monomethylpentoses) is becoming of increasing importance.

For this purpose a complete methylation of the polysaccharide is necessary. An exhaustive methylation with dimethylsulfate is impossible. However, by adaptation of a method developed by White, Morrison & Anderson (3), a thorough methylation is possible. In the original method alkoxides were formed in liquid ammonia and alkylated with alkyl halides. The process has been extended to the simple sugars by Muskat (4) and to the polysaccharides by Freudenberg & Rapp (5).

Measurements on the kinetics of hydrolysis in connection with optical rotation (6) have shown that in cellulose the  $\beta$ -linkage (cellobiose linkage) alone exists, at least no other type of linkage can occur in greater proportion than one in fifty or one in a hundred. The

results with starch are not so restricted. In this case a maximum of one other linkage type can exist in every twenty to twenty-five maltose linkages.

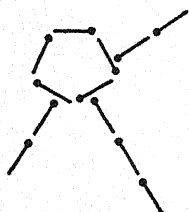


Fig. 1

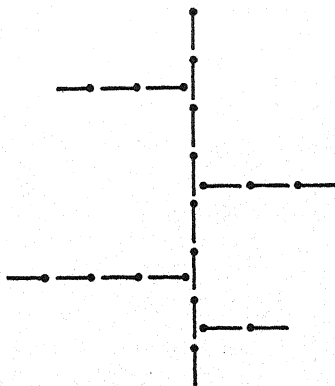


Fig. 2



Fig. 3



Fig. 4

At the present time the following types of polysaccharides are recognizable:

- a) Ring with branched chains: levulans, probably starch, glycogen (Figure 1).
- b) Open branched chains: possibly starch, glycogen (Figure 2).
- c) Open chains without detectable branching: cellulose, possibly chitin and pectin (Figure 3).
- d) Ring without branched chains: Schardinger dextrans from starch (Figure 4).

The number of units varies between a few units (five in crystallized Schardinger  $\alpha$ -dextrin) and hundreds or thousands of units as in cellulose.

#### POLYSACCHARIDES DERIVED FROM SINGLE MONOSES

*Cellulose.*—The question of the chain length in cellulose appeared to have been settled for a time after it had been shown that the length determined by the end-group method [determined as tetramethylglucose (7)] was apparently in agreement with the length of the crystallites in native cellulose (600Å) (8, 9). Both results corresponded to a chain length of from 120 to 250 glucose units as did also

the determination of free aldehydic groups by reduction of the copper or hypiodite solution.

Staudinger (10) has opposed this conclusion. He assumes from his rule of polymeric analogy (see p. 84) that cellulose is not associated in dilute solution but rather dispersed in single molecular chains. Under these conditions Staudinger derived from viscosity measurements a degree of polymerisation of from one to two thousand. Furthermore Hess & Neumann (11) found that intact cellulose after being methylated yielded no tetramethylglucose, or so little that it could not be detected. These authors suggest that the cellulose chain consists of thousands of  $C_6$ -units.

Freudenberg & Plankenhorn (12) found that extremely small amounts of tetramethylglucose and no dimethylglucose are produced from trimethylcellulose. It has been shown by Freudenberg, Plankenhorn & Boppel (13) that the present process of determining end groups is insufficient to estimate the number of glucose units in the chain, which, in cellulose, is of the order of magnitude of a hundred or a thousand. Since several per cent of the methylated substance is lost by the decomposition of trimethylcellulose there is no point in drawing conclusions as to the length of the chain from the amount of tetramethylglucose found, especially when only a fraction of a per cent of it is obtained. Schmidt (14) found one carboxyl group in cellulose for every ninety-six glucose units. Since the carboxyl group was split off by treatment with cold aqueous calcium bicarbonate solution and has been insufficiently characterized, it is doubtful if this observation has much significance for determining the length of the chain.

The condition of native cellulose has been further investigated by Meyer (15). He inferred that adjacent chains are rotated  $180^\circ$  with respect to each other, *i.e.*, in adjacent chains the aldehyde groups are at opposite ends of the units. Hydrated cellulose is recognized as a dimorphous form of native cellulose (16).

Contrasted with the older conception that the length of the crystallites agrees with the length of the chain molecule the assumption has recently been made that the crystallites do not exist in an isolated condition, but rather constitute segregated crystallized regions in the cellulose skeins (17, 18, 19). Thus a chain molecule could project from both ends of the crystallized region.

The physical state of dissolved cellulose, especially the size of the dissolved molecules, has been vigorously discussed within the past few years.

In conformity with the determination of end groups the previously mentioned molecular weight determination by viscosity measurement leads to the belief that the degree of polymerisation of cellulose is of the order of magnitude of a thousand. The evidence may be divided into two parts (20): the determination of the size of the particles; the establishment of the fact that the particles consist of single molecules.

The size of the particles has been determined by osmotic, ultracentrifugal, and viscosity measurements. The determination from viscosity measurements rests upon Staudinger's rule which states that in a polymeric homologous series the degree of polymerisation is proportional to the specific viscosity.

For this purpose Staudinger prepared fragments of different average degrees of polymerisation. The chain lengths (molecular weights) of the smaller fragments were determined from chemical properties—reduction of copper or iodine solution; determination of end groups. The values for the larger fragments were extrapolated from the latter measurements. These determinations alone do not permit a differentiation between single and associated molecules.

A differentiation, however, was made possible through the utilization of the method of polymeric analogy. When a series of polymeric homologous particles was subjected to acetylation and de-acetylation, methylation, nitration, etc., the size of the particles remained constant as determined by viscosity and other characteristic measurements. Thus either the same degree of association was retained during these transformations; or the particles, even the largest, consisted of single molecules. As association forces of the necessary strength are hard to conceive of, it is assumed that the particles consist of single molecules.

According to Staudinger the rod-shaped form of the chain molecules in native cellulose must be retained even in solution. The precipitation of the polysaccharide by acidification, even from dilute cellulose solutions, in at least a partially crystalline form (21–25) could not otherwise be explained, unless at the exact moment of precipitation the rod-shaped forms are again arranged to crystallites. Thus the assumption that cellulose in solution is divided into single macromolecules leads to further difficultly conceivable conclusions.

Lieser (23) is opposed to this conception. Through extensive experiments on the constitution of cellulose solutions he has been led to believe that the crystallites or crystalline regions are partially retained

in solution and therefore reappear upon acidification.<sup>1</sup> If this simple conception is accepted then difficulty is experienced in understanding the phenomenon of polymeric analogy. An attempt to reconcile this contradiction has been made in the suggestion that the thread-like molecules of cellulose could be mechanically knotted in certain places and that thereby the arranged regions could withstand the alterations to which they are exposed during the experiments on polymeric analogy (26).

*Starch and glycogen.*—Starch consists of different components, the two main fractions of which are the soluble amyloamylose and the insoluble amylopectin. There is no longer evidence that amyloamylose constitutes the inner part of the starch kernel, or amylopectin the hull substance. This view has been discarded as well as the opinion that accessory groups such as phosphoric acid are responsible for the difference.

Starch, much more than cellulose, appears to consist of polymeric homologous components of widely different molecular size. The older assumption (27) of a branched starch molecule is confirmed by the following determinations. Of every twenty glucose units one is an end group (yields tetramethylglucose) and one is in a branching position (yields dimethylglucose). After the exhaustive methylation of starch was made feasible, the proportion of these groups was shown to be approximately equal (28). Staudinger & Eilers (29) from viscosity measurements assumed a strongly convoluted form for the starch particles which results from branched chain molecules (Figure 2) (30). Furthermore, the constitution of the so-called Schardinger dextrans has been clarified (31, 32). These highly important crystallized dextrans are formed by the action of *Bacillus macerans* upon starch. In all probability the  $\alpha$ -dextrin is a cyclic pentaose containing five maltose bonds (Figure 4). Such a system, which is probably preformed in the starch (33), necessitates the assumption of side chains (Figure 1).

Myrbäck (34) has recently emphasized the older conception (35)

<sup>1</sup> In this discussion I have avoided the misleading word "micelle" which, for example in the colloid chemistry of soap, is connected with the idea of arranged bundles of small molecules. Neither Lieser nor others mean, in the case of cellulose, associations of small molecules. Many controversies arise from the fact that some authors use the word micelle as meaning arranged associations of small molecules whereas others employ it in the sense of arranged associations of small or large molecules [cf. Frey-Wyssling (19)].



that the decomposition of starch with  $\beta$ -amylase proceeds from the end of the chains (or side chains) from which the enzyme splits off one maltose after another, until a different linkage type or phosphoric acid group, etc., is encountered upon which the enzyme has no effect.

The Schardinger dextrins are produced from starch in a yield ranging from a few per cent up to approximately 14 per cent. If the latter figure be considered as the maximum, then such a dextrin, consisting of five or six units ( $\alpha$ - and  $\beta$ -dextrin), must occur in every thirty-five to forty-five glucose units. Inasmuch as two end groups are present in this figure one could assume a five- or a six-membered ring with two side chains containing a total of thirty to forty units. Thereby the difficulty arises that no arrangement can be conceived (with the exception of a true diacetyl linkage which has not been proved) in which more than one Schardinger-dextrin type exists and thus starch could possess a degree of polymerisation of only thirty-five to forty-five. On the other hand the determination of the size of particles which, according to the rule of polymeric analogy, should be equal to the molecular size results in a degree of polymerisation of the order of magnitude of a thousand or more. The contradiction could be harmonized by the assumption that the forces of association are superimposed by a mechanical tying together of the single molecules to difficultly dispersible aggregates.

Through the action of  $\alpha$ -amylase upon starch and glycogen, the  $\alpha$ -form (decreasing positive rotation) of oligosaccharide, which is of approximately the same size as the hexaoses, is liberated.  $\beta$ -Amylase produces maltose which at first appears in the  $\beta$ -form (increasing positive rotation). In the latter case, as is generally recognized, Walden inversion occurs at the cleavage position. The earlier assumption that  $\alpha$ - and  $\beta$ -linkages alternate in starch has been disproved by the kinetics of starch hydrolysis. A survey on the biochemistry of starch has been given by Hanes (36) (for the chemistry of starch cf. 30).

Glycogen, upon treatment with *Bacillus macerans*, also yields crystallized dextrins. The fact that these dextrins yield colored addition products when treated with iodine indicates that they are preformed in starch and glycogen.

An end-group determination (tetramethylglucose) shows the "chain length" of glycogen to be approximately twelve to eighteen units, *i.e.*, smaller than starch. This means that the individual branches of the molecule are shorter than in starch.

*Pectin* (37, 38).—Even though decisive proof is lacking, it is generally denied that the galactose and arabinose units present in pectin preparations are chemically linked to the galacturonic-acid complex discovered independently by Ehrlich and by Suarez. Doubtless, pectin consists chiefly of polygalacturonic acids, part of which are esterified with methylalcohol and part of which are present as calcium and magnesium salts. Link's (39) hypothesis that the chief constituent of pectin is a chain-membered polysaccharide consisting of partially esterified units of galacturonic acid has been recently verified by Henglein & Schneider (40; cf. also 41). It is uncertain whether or not acetyl linkages may be included in this conception. Levene & Kreider (42) have explained the linkage type. It corresponds to the cellulose linkage.

*Alginate acid* (43).—This acid, derived from algae, consists entirely of mannouronic acid groups. It possesses a marked tendency to swell, is soluble in alkali and may be precipitated with acids.

#### POLYSACCHARIDES DERIVED FROM DIFFERENT SUGAR TYPES.

##### MIXED POLYSACCHARIDES

The majority of those polysaccharides which are classified under the indefinite terms of hemicelluloses, vegetable gums and mucilage belong to this group (38). The polysaccharide portion of the glycoproteins as well as the serologically active specific polysaccharides must be included here also. The treatment of the latter substances as well as the sulfuric acid esters of polysaccharides must be dealt with at some later time.

*Mixed polysaccharides containing no uronic acid.*—Based on the preliminary work of Tollens and others, Haworth and his coworkers have recently investigated the xylan from esparto (44). This xylan does not reduce Fehling's solution. Through the action of mineral acid it is decomposed principally to xylose and a small amount of arabinose. Haworth and coworkers assume the presence of chains containing approximately eighteen xylose units which are connected at the hydroxyl group in position four and contain an arabofuranose residue on one end.

According to this observation it would seem doubtful that the other xylans, upon which but little research has been done, consist solely of xylose. Such xylans are very widespread in lignified cells. Beech wood contains 16 per cent and wheat straw 25 per cent of xylan. Coniferous woods contain only small amounts of xylan. Enzymes which

are capable of splitting off xylan groups are found in the digestive fluid of the vineyard snail and in germinating barley grain (45).

Galactoaraban and galactomannan—a polysaccharide consisting chiefly of galactose and approximately one-seventh arabinose is found in larch wood (38). A galactomannan (1:1) is present in lucerne seeds (46).

Glucomannan (or Konjac mannan)—a mixed polysaccharide which consists of one glucose and two mannose units. Nishida & Hashima (47) have obtained the acetate of the triose by acetolysis and from this the free triose in crystalline form. The triose is a mannosido-mannosido-glucose in which two 1:6 linkages are present.

*Polysaccharides containing a uronic-acid component.*—In many cases it is possible to differentiate between two types of hemicelluloses which contain uronic acid (38): (a) glucose series—those which contain *d*-glucose (often lacking), *d*-glucuronic acid (small amount), and *d*-xylose (large amount); (b) galactose series—those which contain *d*-galactose (large amount), *d*-galacturonic acid (lesser amount), and *l*-arabinose (often lacking). Glucose and galactose are converted in the plant into uronic acid which, in turn, yields the corresponding pentoses (*d*-xylose and *l*-arabinose). Up to the present time man-nuronic acid has been found only in algae. Members of the glucose series include the hemicelluloses of the corncob, cottonseed hulls, oat hulls and spruce wood.

Vegetable gums are sticky exudates and frequently of pathological origin. They ordinarily occur as calcium or magnesium salts of uronic acids which, in turn, are linked to polysaccharides. Gum arabic, from tropical acacias, contains galactose and glucuronic acid in addition to arabinose and rhamnose. A crystalline disaccharide composed of galactose and glucuronic acid was found in the degradation products. It has also been synthesized by Hotchkiss & Goebel (48). Under certain conditions an electro-osmotically purified solution of the polysaccharide can serve as a substitute for blood plasma. Up to 70 per cent of cat's blood may be so substituted.

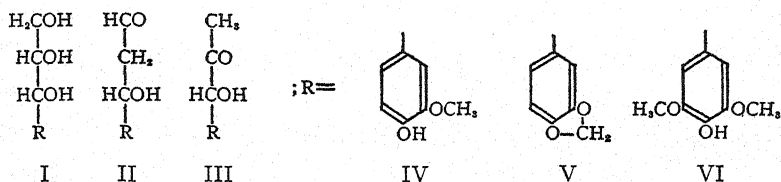
Cherry gum, tragacanth gum, linseed and mustard-seed mucilage and many other polysaccharides belong to this class (38).

## LIGNIN

The last attempt to present a coherent constitutional scheme for lignin was made more than five years ago (49). Although the scheme advanced at that time has been criticized in certain respects, a more

satisfactory picture has not yet been suggested.<sup>2</sup> The observational material accumulated in the interim has confirmed the fundamental principles of these earlier structures, although it has necessitated some modifications. A descriptive survey of the chemistry of lignin has been given by Phillips (50) and by Hägglund (51).

Lignin may be regarded as a product resulting from the etherification and condensation of the following and similar units (52).



In lignin from the beech (*Fagus silvatica*), between a third and a half of the R-groups are of the syringic group VI (53), whereas in spruce lignin this constituent occurs to the extent of only a few per cent.

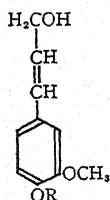
Unless otherwise noted all references to lignin apply solely to the product obtained from spruce (*Pinus excelsa*).

#### RELATED PLANT SUBSTANCES

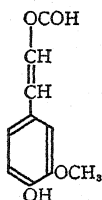
P. Klason (54) was the first to recognize the close relationship existing between lignin and the glucoside of coniferyl alcohol (VII) which is present in the cambial sap of the spruce. However, he was unable to prove such a relation. Lignin differs fundamentally from the easily formed polymerisation or condensation products of coniferyl alcohol (*cf.* pp. 94, 107), in that every conversion product of coniferyl alcohol possesses free phenolic groups whereas lignin does not, or only to a small extent. In a later paper P. Klason (55) suggested that coniferyl aldehyde may be the basic unit in lignin. The same difference relative to phenolic groups exists between lignin and the lignanes (56) as between lignin and condensation products of coniferyl alcohol. Lignanes (skeleton formulae X and XI) are resinous natural products derived from coniferyl alcohol or certain of its oxidation products

<sup>2</sup> Although the author would like to discuss critically certain controversial points of lignin structure, it is more desirable in such a paper as this to give a coherent picture of the whole subject. In order to do this the author has put forward what seems to him the most logical conception of lignin chemistry. He hopes that it may stimulate those who do not agree to propose another more constructive solution to this problem.

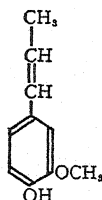
such as coniferyl aldehyde and ferulic acid (VIII) or the related isoeugenol (IX). The nuclei (V) and (VI) also occur within the lignane group.



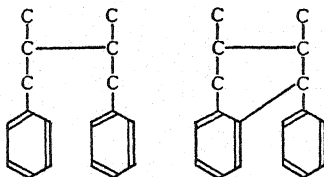
VII. Coniferyl-alcohol (R = H).  
Coniferin  
(R = Glucose residue)



VIII. Ferulic acid



IX. Isoeugenol



X

XI

Carbon frame of the  
lignanes

The substance XI is to be regarded as a condensation product of the compound X. Many of these lignanes are easily polymerized or condensed to amorphous products as is the case with coniferyl alcohol. In the lignanes and their condensation products, as contrasted with lignin, the phenolic group in the para position is not substituted.

#### STATE AND LINKAGE OF LIGNIN IN WOOD

At the present time unaltered lignin cannot be separated from the other constituents of wood with which it occurs. There are three reasons for this:

a) Part of the lignin is combined with hemicelluloses or simple sugars through a glucoside linkage which possibly involves the secondary carbinol groups. Only a partial separation of this sugar constituent results from the use of enzymes. A complete separation necessitates an acid hydrolysis which, however, alters lignin.

b) Lignin exists in different condensation steps according to the age and origin of the wood. Highly polymerized sugar-free constitu-

ents occur together with lower polymers containing sugar. Treatment with acid, in order to separate the sugar, condenses the smaller parts into larger ones.

c) Cellulose, the chief constituent of wood, does not appear to be chemically linked with lignin. Nevertheless, it is impossible to extract the cellulose from wood by use of its specific solvents inasmuch as a swelling of the chain molecules occurs in copper, viscose, or acetate solution which prevents its entrance into the solution by diffusion (57).

Closely connected with the structural relations described under *a* and *b* is another phenomenon. A small proportion of spruce lignin and a large proportion of beech lignin are at first dissolved in highly concentrated hydrochloric acid. These fractions are then precipitated due to the hydrolytic cleavage of sugar and the condensation of the lignin particles. According to Hilpert (58) lignin is formed from carbohydrates during the isolation processes. This opinion must, however, be discarded.

#### ISOLATION

The isolation of lignin involves two interdependent reactions: the separation of the lignin from the carbohydrates (when joined); and the dissolution of the carbohydrates. The most vigorous methods consist in the use of highly concentrated mineral acids whereby the polysaccharides are decomposed. Willstätter & Zechmeister (59) used cold 40 per cent hydrochloric acid; others have used fairly concentrated sulfuric acid (60), liquid hydrogen chloride (61), liquid hydrogen fluoride (62), a mixture of hydrochloric acid with sulfuric acid (63), or with phosphoric acid (64).

Contrasted with the "acid-lignins" (*i.e.*, lignins prepared with mineral acid) is the so-called "cuproxam-lignin" (65, 66). The preparation of the latter requires the use of dilute mineral acids (for example 1 per cent sulfuric acid at 100°) or hot oxalic acid in order to hydrolyze the lignin-carbohydrate linkage and to decompose the easily hydrolyzed polysaccharides. The difficultly hydrolyzable polysaccharides, including cellulose, are then dissolved from the loosened structure through application of a solution of copper oxide in ammonia. A part of the less polymerized forms of lignin which are linked to sugar can be removed previous to either of the above-mentioned treatments by cold alkali or by organic solvents such as cold formic acid (67). This treatment is of little importance for spruce as only small amounts of the wood are soluble. For the beech or other

hard woods, the soluble part is very large. If the soluble portion is hydrolyzed afterwards, an insoluble lignin is formed, which no longer shows the morphologic structure of the tissue, as does that part of lignin which has never been dissolved. On the other hand, the chemical difference between the two lignin fractions is very small. The cuproxam-lignin of the spruce differs from the corresponding acid-lignin in its greater solubility in acid sulfites, its higher content of bound formaldehyde, and its lower average degree of polymerisation.

Whereas acid-lignin may be utilized for some experiments, especially for preliminary tests, cuproxam-lignin, exclusively, should be used for the more exact experiments. Wherever possible, it is preferable to carry out reactions with the lignin *in situ* in presence of the polysaccharides, namely in the wood. Wood which has been treated with methylating or ethylating agents can be used for isolation of lignin derivatives, although the derivatives cannot be isolated by the use of solvents alone.

#### PHYSICAL PROPERTIES

Lignin is altered by all the procedures which are able to dissolve it from wood, either completely or partially, as for example by extraction with alkali or with methylalcoholic hydrogen chloride, or by cooking with acid sulfite. If such preparations are employed in experimental tests, the alterations must be taken into account. Carefully prepared spruce lignin, as well as the insoluble part of hardwood lignin, possess the morphologic structure of the cell (68). Extended hollow spaces, resulting from the removal of submicroscopic skeins or fibrils may be recognized by the double refraction observed under a polarizing microscope. That this is double refraction caused by morphological structure is shown by the fact that as the refractive index of the immersion liquid increases the double refraction becomes weaker, disappears with iodobenzene ( $n = 1.62$ ), and then reappears with liquids having a larger index of refraction. By this the refractive index of spruce lignin has been determined as 1.61, which is in accordance with the aromatic nature of lignin. Furthermore, the position of the hollow spaces of the cellulose skeins with respect to the fiber axis may be determined in the polarizing microscope as being predominately tangential in the primary layer and axial in the secondary layer (68).

Lignin possesses the following characteristics: x-ray analysis proves it to be amorphous in form; its swelling capacity is limited;

mechanical properties are equal in all directions; and its tensile strength is extremely small. Cuproxam-lignin from the spruce is light tan, whereas that of the beech is even lighter colored. Acid-lignins, especially those obtained from hard woods are darker colored. The layer thickness of lignin which has been dissolved in the form of a salt of lignin-azobenzenesulfonic acid is  $20\text{\AA}$  (69). Thus it constitutes a three dimensional picture. Lignosulfonic acid, through the position and intensity of its spectral absorption bands, is characterized as being an aromatic substance (70). For the most part it is incapable of being dialyzed. It is of high molecular weight, just as is free lignin. Thus, satisfactory molecular weight determinations are impossible.

#### ANALYTICAL EVIDENCE

Spruce lignin which has been thoroughly dried shows the following percentage composition: C, 65 to 66; H, 6.1;  $\text{OCH}_3$ , 15 to 16;  $\text{O}_2\text{CH}_2$ , 4; OH, 10;  $-\text{O}-$ , 9;  $\text{C}-\text{CH}_3$ , 2.7. Cuproxam-lignin contains 1 to 2 per cent of nitrogen as a result of the preliminary treatments (71). In the analytical results this nitrogen is computed together with oxygen. Hydrochloric acid-lignin contains up to 3 per cent chlorine which must be considered in the calculation of the analyses.

The oxygen present in lignin cannot be completely accounted for in the methoxyl, methylenedioxy, and hydroxyl groups. The excess (9 per cent) of oxygen may be regarded as combined in ether linkages, the extent of which is approximately equivalent to the methoxyl and hydroxyl groups present. The methoxyl group occurs primarily in the guaiacol group (IV) and to a very small extent in syringic residues (VI) (72).

The occurrence of additional aliphatic methoxyl groups is possible to only a very limited extent [possibly 1 to 2 per cent (73)]. The formaldehyde is obtained from aromatic methylenedioxy groups [condensed piperonyl groups (V), *cf.* p. 96]. It can be determined as formaldehyde by direct cleavage with mineral acids or as acridan by reaction with aniline (74). Cleavage of the methylenedioxy groups in lignin also occurs through treatment with metallic potassium in liquid ammonia. Piperonylic acid and other aromatic methylenedioxy compounds behave in the same way. The hydroxyl groups belong principally (6 to 8 per cent) to secondary carbinols which can be methylated, toluenesulfonated, and acetylated. A smaller number are tertiary and can be acetylated only. The  $\text{C}-\text{CH}_3$  group (2.7 per cent) yields acetic acid (found 6 per cent acetic acid) by oxidation

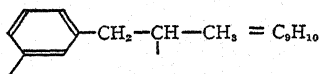


with chromic acid. In amount it is approximately equivalent to the tertiary carbinol (75).

As compared with spruce lignin, beech lignin contains a few per cent less carbon (C, 60.5; H, 5.8) and more methoxyl (21.5) (76). The content of methylenedioxy groups is smaller (about 2 per cent), whereas C—CH<sub>3</sub> (7 per cent) is much higher than in spruce (53). These data apply only to the insoluble portion of beech lignin. The divergences from spruce lignin may be partially explained by the higher content of the syringic component (VI).

#### LIGNIN AS A DERIVATIVE OF PHENYLPROPANE

If all of the methoxyl, hydroxyl, and ether groups were substituted by hydrogen, a ratio of carbon to hydrogen of 1:1.1 would be obtained for the resulting compound. This ratio may be adequately explained by assuming the following simplified chain member:

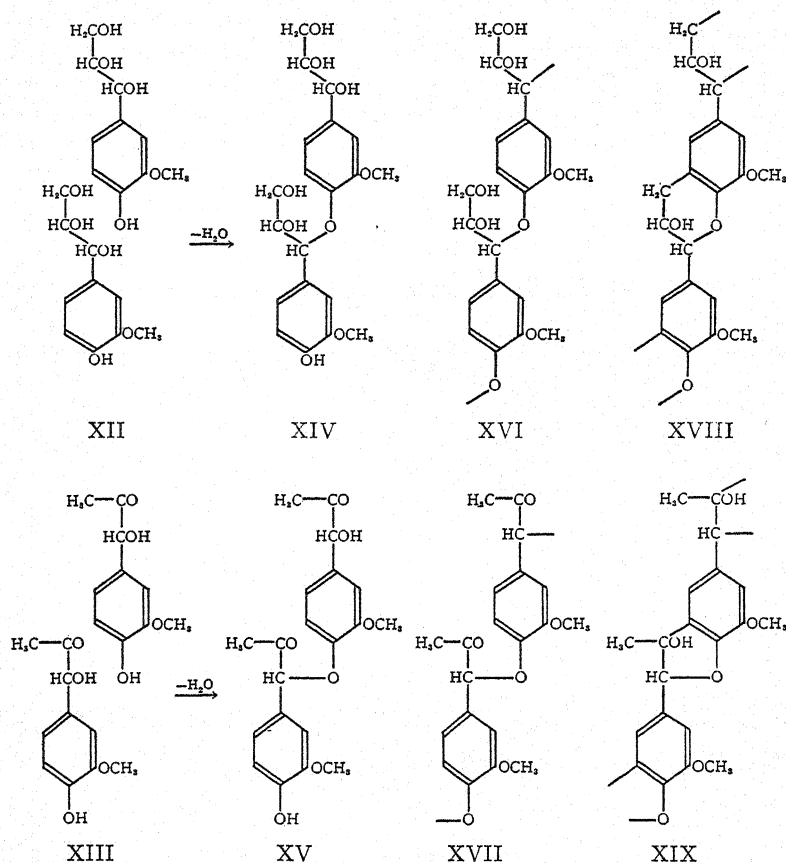


The basic type structure of lignin is in accord with a structure composed of benzene homologues. Lignin chemistry can be best explained by the assumption of the above-mentioned phenylpropane group as the fundamental type-unit of structure.

#### REGARDING THE CONSTITUTION OF SPRUCE LIGNIN

Of the nine phenylpropane derivatives which can result from combination of units I–VI chosen from numerous possibilities, only two will be discussed, namely guaiacyl glycerine, XII, and acetyl guaiacylcarbinol, XIII. The central idea in the conception of lignin structure as presented here is that lignin is composed of similar units which unite with each other (52) as do the amino acids in proteins or the monoses in polysaccharides. We assume that the units are connected through an ether linkage between phenol-hydroxyl and the carbinol group of the side chain.

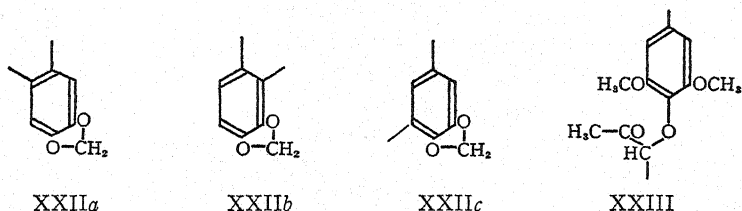
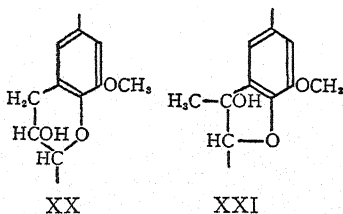
The double units XIV and XV, as well as the single units XII and XIII, contain a carbinol group on one end and a phenol group on the other. Hence they are capable of building triple and larger units in accordance with the same linkage principle. Such aggregates are represented by the general structures XVI and XVII. Double or triple units have not yet been isolated.



Furthermore it is assumed that molecular portions, such as XVI and XVII, can undergo condensation whereby chroman (XVIII) or furan (XIX) rings are produced.

The constantly recurring middle portion in such formulae are represented by the simplified schemes XX and XXI. In addition to these condensed systems, noncondensed ether systems corresponding to formulae XVI and XVII are also assumed. The few end groups which are present appear to exist principally in the form shown by formulae XIV and XV, because oxidation subsequent to methylation yields only a few per cent of veratric acid (XXIV). Those groups which split off formaldehyde are intermediate members within the chains, *e.g.*, formula XXII *a* or *b*.

Whether or not this arrangement for the methylenedioxy groups is correct has yet to be proved. Formaldehyde is not obtained from a lignin (Scholler-Tornesch process) which is isolated by treatment with 0.5 per cent sulfuric acid at 180°. Subsequent to methylation and oxidation, it yields no isohemipinic acid (XXVI). Thus, such an arrangement as XXII *c* is unlikely.



The pyrogallol component in beech lignin exists as a symmetrical dimethylether in which the middle hydroxyl group is etherified. Probably, units like that shown in formula XXIII occur. The small amount of the pyrogallol component which occurs in spruce lignin may also have the same arrangement.

This conception is in agreement with the following analytical evidence: five out of every eight units in spruce lignin belong to types like XX or XXI (this applies also to the corresponding noncondensed forms); two units contain methylenedioxy groups; and one unit, or less, is of the pyrogallol type XXIII. These forms are mixed so that part of the benzene rings could condense with more than one unit, thus producing two or three dimensional structures. The average "molecular weight" per unit is between 180 and 190, *i.e.*, 185. This figure is somewhat larger than has been accepted up to the present time. The true molecular weight of the polymer is a multiple of this.

#### FUNCTIONAL DERIVATIVES

The methoxyl content of spruce lignin is nearly doubled (to 29 per cent) by methylation with dimethylsulfate. The methoxyl content of cuproxam-lignin when treated with diazomethane is increased by approximately 2 per cent. Only a small part of this methoxyl can be

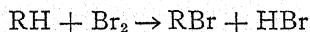
aromatic, *i.e.*, linked to benzene rings. It is impossible with diazomethane to make a sharp distinction between phenolic and alcoholic hydroxyl groups.

Methyl lignin (29 per cent  $\text{OCH}_3$ ), after acetylation, contains acetyl groups corresponding to 2.4 per cent of tertiary hydroxyl (77). Accordingly lignin acetylated by acetic anhydride and pyridine has a higher acetyl content than would be expected from the methylation results. The majority of the hydroxyl groups react with toluenesulfonyl chloride (78) and benzyl chloride (79). Nevertheless the extent of this reaction is less than with dimethylsulfate. It is evident from the behaviour with hydrazine that the toluenesulfonyl groups are connected to carbinol, rather than to phenol groups (78). Methylation, benzylation, acetylation, or toluenesulfonation of lignin results in compounds of unaltered insolubility in organic solvents. This is especially remarkable in the case of toluenesulfonic ester because in the formation of this compound the weight of lignin is doubled. Carbonyl groups, if present in spruce lignin, are present in very small amounts (51). Carboxyl groups are also undetectable.

#### SUBSTITUTION PRODUCTS

By treatment with alcoholic mercuric acetate it is possible to substitute a hydrogen atom in the benzene ring by the  $\text{HgOCOCH}_3$  group. On the average one molecule reacts per unit of methyl lignin (80). Mercury can be replaced by iodine. The iodine substitutes in the aromatic nucleus (80) and forms iodomethyl lignin. Preparations which contain more than 40 per cent mercury or 35 per cent iodine do not differ in external appearance from unsubstituted methyl lignin.

The bromination proceeds less smoothly (73) for by this treatment a portion of the methyl is split off, as happens also from vanillic acid. However, the experiment clearly shows that a substitution according to the following scheme occurs:



The main reaction consists in the substitution of approximately one bromine atom per unit. In the experiment, it is necessary to retard the oxidation by using bromine dissolved in an excess of hydrobromic acid.

During nitration it is more difficult to prevent oxidation. Nevertheless it has been possible to prove that by the action of nitrogen dioxide on methyl lignin the main reaction is a substitution (81) and that its extent is approximately one nitro group per unit.

If nitro lignin is brominated the bromine will substitute additional

hydrogen atoms in the ring. Bromolignin when nitrated behaves in an analogous manner. From the extent of substitution, it may be shown that from 1.5 to 2 hydrogen atoms per unit have been substituted (81).

#### DIRECT DEGRADATION

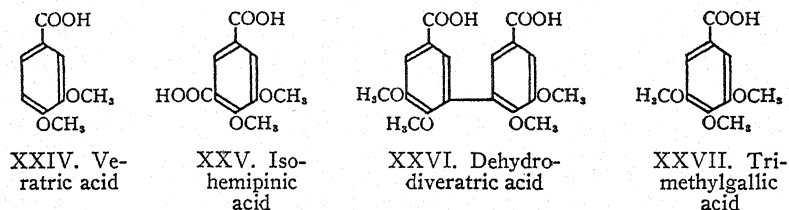
By fusion with potash at 250°, lignin is not sufficiently decomposed to give recognizable degradation products. At approximately 270°, an exothermic reaction occurs by which the framework is broken up. Aside from oxalic acid, a 10 per cent yield of protocatechuic acid is obtained. The latter is isolated in the form of veratric acid. No better yields are obtained from eugenol or "polymeric" coniferyl alcohol by potash fusion (77). Potash fusion of beech lignin at 210–215° yields primarily gallic acid (4 to 5 per cent) (67) which is isolated as trimethylgallic acid. If fusion is carried out at 270° from 3 to 3.5 per cent each of protocatechuic and gallic acid are obtained. Due to loss in separation these figures constitute minimum values (53). Direct, careful oxidation of spruce lignin yields approximately 20 per cent vanillin (82). If powdered wood is used instead of lignin a better yield (calculated on lignin content) is obtained. Furthermore, a small amount of vanillic acid results. Bromolignin yields 6 to 8 per cent of 6-bromovanillin (82). The latter result constitutes a proof that the phenolic group was etherified at the time the bromine reacted, because only alkyl or acyl derivatives of vanillin or vanillic acid are brominated in position-6; with unsubstituted hydroxyl groups, position-5 reacts.

Spruce lignin, methylated with diazomethane or dimethylsulfate, yields 1 or 2 per cent veratric acid by oxidation with permanganate. If powdered wood is treated with diazomethane (whereby it is bleached) (83) a better yield (4 per cent calculated on lignin content) (84) is secured by oxidation with permanganate. Inasmuch as approximately one-third of the veratric acid is further decomposed by the oxidation process, we may assume that the total amount of this acid is 6 per cent of the lignin content. This means that on the average every sixteenth unit possesses a free guaiacyl residue in the end position, and that lignin as it exists in wood contains approximately 0.6 per cent free phenolic groups. However, it is probable that most of the veratric acid originates from the low molecular preliminary stages in lignin formation which are for the most part lost by the isolation of lignin. Thus, no far-reaching conclusion can be drawn from this evidence.

## DEGRADATION FOLLOWING CLEAVAGE OF ETHER BONDS

*Alkali.*—Spruce lignin (67, 84), or better the wood itself, subsequent to methylation with diazomethane, 90 minutes cooking with 70 per cent potassium hydroxide at 165 to 170°, methylation with dimethylsulphate, and oxidation with permanganate yields (calculated from lignin content): 20 to 21 per cent veratric acid (XXIV), 6 to 12 per cent isohemipinic acid (XXV), 2 to 3 per cent dehydrodivertrac acid (XXVI), and traces of trimethylgallic acid.

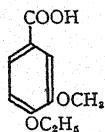
This alkali treatment differs from the potash fusion (cf. p. 98) in that the carbon framework remains essentially unaltered, the rupture occurring chiefly at ether bonds and at the oxygen linkages of the heterocyclic rings. Free phenolic groups must be protected by methylation before the subsequent oxidative degradation is undertaken.



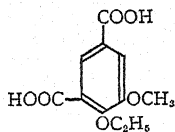
Dehydrodivertrac acid is formed by a secondary oxidation during the cooking. This oxidation can be prevented by the complete elimination of molecular oxygen. At the same time trimethylgallic acid occurs in small amounts (a fraction of one per cent).

From the qualitative as well as from the quantitative viewpoint, these determinations constitute the chief support for the schemes outlined on p. 94 (formulae XVI–XXIII). The alkali effects the splitting of the ether linkages shown in XVI and XVII as well as opening of rings in such chain members as XVIII and XIX. In both the latter cases isohemipinic acid is produced, whereas veratric acid results from the following three sources: units in end positions; rupture of ether linkage (XVI and XVII); and further, degradation of condensed systems (XVIII–XXIX). Trimethylgallic acid is produced, as shown by the corresponding ethylation experiment (see below), from an arrangement similar to XXIII.

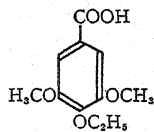
Spruce wood, subsequent to treatment with diazoethane, decomposition with alkali, ethylation, and oxidation, yields the corresponding ethyl ether acids (XXVIII and XXIX).



XXVIII. Ethyl-ether-vanillic acid



XXIX. Ethoxy-methoxy-isophthalic acid



XXX. Ethyl-ether-syringic acid

However, the yields are much worse because the ethyl ethers of these acids are more rapidly destroyed by permanganate than the corresponding methyl ethers. The significance of these ethyl ether acids is that the ethyl groups substitute the opened ether bonds, whereas the methoxy groups remain as they were in lignin.

In order to estimate quantitatively the proportions of the acids present in the original lignin, the decomposition of the acids themselves was determined by treating with permanganate in the same manner as the decomposed alkylated lignin. Percentages recovered and approximate conversion factors for lignin analysis are here listed (74):

	Percentage	Factor
Veratric acid .....	72	1.4
Isohemipinic acid .....	11	9.0
Dehydrodiveratric acid .....	97	1.0
Trimethylgallic acid .....	57	1.75
Piperonylic acid .....	0	...

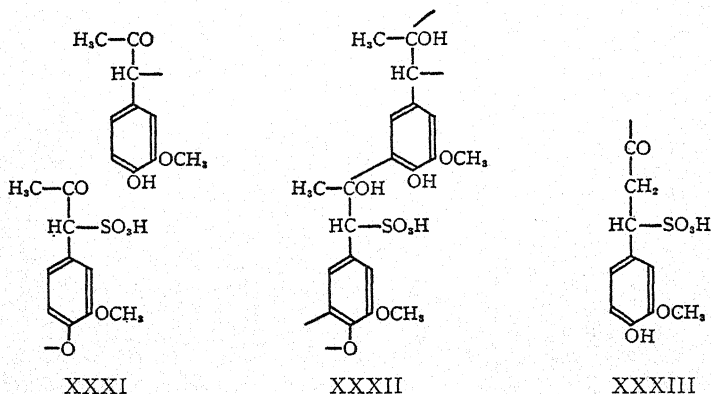
These figures, naturally, are only approximate, but when the factors are applied to the analysis of spruce lignin, the content of isohemipinic acid found is about 80 per cent and that of veratric and dehydrodiveratric acids together about 32 per cent. If from three lignin units (mol. wt.  $3 \times 185$ ), one yielded veratric acid (mol. wt. 182) and two yielded isohemipinic acid (mol. wt. 226), then 33 per cent of the original weight of lignin would be veratric acid and 81 per cent would be isohemipinic acid. Since this estimate agrees with the observed amounts of methylated phenolcarboxylic acids obtained, the conception is substantiated that lignin consists entirely of like those units outlined on p. 94 and that these are for the most part united as shown in schemes XVIII and XIX (66). Units in which methylenedioxy groups (XXII *a* and *b*) are present, probably produce veratric acid after formaldehyde has been split out and decarboxylation has occurred. The experiments with the substances used as models outlined on p. 106 support the above result. The corresponding experiments with beech wood produce less favorable results because of the more difficult separation of the trimethylgallic acid (53).

	Percentage Found	Percentage Corrected
Veratric acid .....	5.0	7.0
Isohemipinic acid .....	1.5	13.5
Trimethylgallic acid .....	7.5	13.0
	14.0	33.5

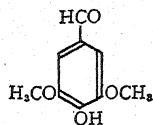
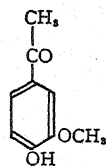
Beech lignin upon ethylation yields the ethyl ether of syringic acid in addition to the ethyl ether of vanillic acid. The methoxy groups remain in the original position in the pyrogallol residue. Symmetrical dimethylpyrogallol and its derivatives are also found in the distillation products of beech wood.

*Bisulfite*.—Cuproxam-lignin can be dissolved by repeated cooking with a sulfite solution. After methylation and oxidation about 3 per cent each of veratric acid and isohemipinic acid are produced from the ligninsulfonic acid (calculated from lignin content). It is easily understandable that dehydrodiveratric acid is not produced. Using the above-outlined method of calculation, it is found that ligninsulfonic acid contains components which yield 4 to 5 per cent of veratric acid and up to 30 per cent of isohemipinic acid. Lignin itself yields only 1 to 2 per cent veratric acid by oxidation following methylation. Thus the phenolic hydroxyl group in the para position in about every third unit is simultaneously freed by the entrance of sulfonic acid.

The ligninsulfonic acid employed contained 5 per cent sulfur. This also means that every third unit has reacted with the sulfite. In formulae XVII and XIX (and correspondingly in formulae XVI and XVIII) the reaction may be explained by assuming that the ether linkages are ruptured by the sulfite treatment, thus forming compound XXXI from XVII and compound XXXII from XIX.





XXXIV. Syringic  
aldehydeXXXV. Aceto-  
vanillone

After methylation the top unit of XXXI yields veratric acid on oxidation, whereas the substance in the corresponding position of XXXII produces isohemipinic acid. The increase in phenolhydroxyl groups in ligninsulfonic acid, as compared with the number in lignin, is very important for the tanning properties. To this must be added the free phenol groups resulting from the cleavage of the greater portion of the formaldehyde from the methylenedioxy groups, for only 0.6 per cent formaldehyde is detectable in ligninsulfonic acid.

If ligninsulfonic acid from cuproxam-lignin is subjected to further sulfite treatment the sulfur content can be raised to 8.6 per cent (85). Following methylation and oxidation such a preparation yields 2.8 per cent veratric acid and 3.8 per cent isohemipinic acid (85). Part of the sulfonic acid groups could have reacted with carbinols which are adjacent to benzene rings (compare XIX). Examples of this type of reaction are given on p. 106.

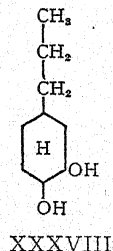
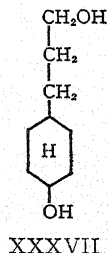
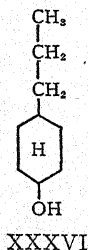
Thus the results of the sulfurous acid treatment would be to rupture the few ether linkages between phenol groups and secondary  $\alpha$ -phenylcarbinols. Oxygen atoms in corresponding rings, secondary or tertiary  $\alpha$ -phenylcarbinols all react in like manner (compare XIX).

Ligninsulfonic acid when heated with dilute alkali at 160° to 180° produces vanillin (86). Under the best experimental conditions, Tomlinson & Hibbert (87) obtained a yield of 6 to 8 per cent of vanillin (calculated from lignin content). Hägglund, together with Bratt (88) and Alvfeldt (89), determined that the yield parallels the content of sulfonic acid groups. Hibbert assumed that vanillin is produced from structures like XXXIII. This conception is in general agreement with the representation here outlined. Hibbert and his co-workers (91) have obtained 2.9 per cent each of vanillin and syringic aldehyde (XXXIV) from beech ligninsulfonic acid. In addition, small amounts of acetovanillone (XXXV) are formed from the ligninsulfonic acids of the beech (0.8 per cent) (90) and also of the spruce (91) (0.3 per cent). Acetosyringone (91) has been prepared in very small amounts from birch ligninsulfonic acid.

*Thioglycolic acid.*—Bror Holmberg's (92) reaction with thioglycolic acid and aqueous mineral acids, whereby the larger portion of soft and hardwood lignins becomes soluble in alkali, can be explained similarly to the reaction of lignin to form the sulfonic acid. The  $-\text{SO}_3\text{H}$  groups in formulae like XXXI and XXXII are to be replaced by  $-\text{SCH}_2 \cdot \text{COOH}$ . The evidence for this conception is found in the results of methylation and oxidation by means of which small yields of veratric and isohemipinic acids are obtained (84). Furthermore, experiments on substances known to contain such linkages support this conception (*cf.* pp. 105, 106).

*Alcohols and mineral acids.*—P. Klason (93) discovered in 1893 that part of the lignin is dissolved if spruce wood is cooked with methylalcoholic hydrogen chloride. Besides lignin, less than half of which is dissolved, the extract contains conversion products of sugar. By methylation and oxidation of the dissolved lignin 3.5 per cent veratric acid and 2.5 per cent isohemipinic acid are produced (66). The reaction can be understood if the sulfonic acid group in formulae XXXI and XXXII is substituted by methoxyl. This may thus be looked on as an exchange of ether radicals accompanied by a partial degradation of lignin molecules. The insoluble portion remaining in wood may have been condensed by the mineral acid. The soluble portion contains more methoxy (20 per cent) than the lignin. The same reaction can be carried out to advantage with benzylalcohol, glycol, dioxane-containing acetals, etc.

Hardwood yields more soluble methanollignin than spruce. Harris, D'Ianni & Adkins (94) carried out a hydrogenation under pressure on a methoxyl-rich fraction from aspen. Besides a great deal of methyl alcohol that originated from methoxyl groups present, both from those introduced by methanolysis and from those originally present, they obtained important cyclohexylpropane derivatives such as those represented by formulae XXXVI to XXXVIII.



The amount of hydrogen absorbed (one mol per 25 to 30 gm. of lignin) corresponded to the expected quantity. If, for example, the hydrogenation product XXXVI resulted from substances XVI or XVII, then one mol of hydrogen would be required for 26 gm. of lignin. In addition, cleavage products of higher molecular weight were produced by hydrogenation. The latter were further hydrogenated and finally yielded hydrocarbons containing eighteen and more carbon atoms and possessing the empirical formula  $C_nH_{2n-2}$ . The conversion of two units of substances XVIII or XIX into a hydrocarbon would actually result in  $C_{18}H_{34}$ .

It may here be mentioned that Phillips & Goss (95) obtained 0.4 per cent dehydroeugenol (1-propyl-3-methoxy-4-hydroxy-benzene) from zinc-dust distillation of lignin from corncobs.

*Hydrazine; potassium in ammonia.*—Wood, as well as cuproxam-lignin, when dissolved in water-free hydrazine and heated to  $140^\circ$ ,<sup>8</sup> loses a large portion of its methoxyl groups. Following methylation and oxidation 6.5 per cent veratric and 2.5 per cent isohemipinic acid were obtained.

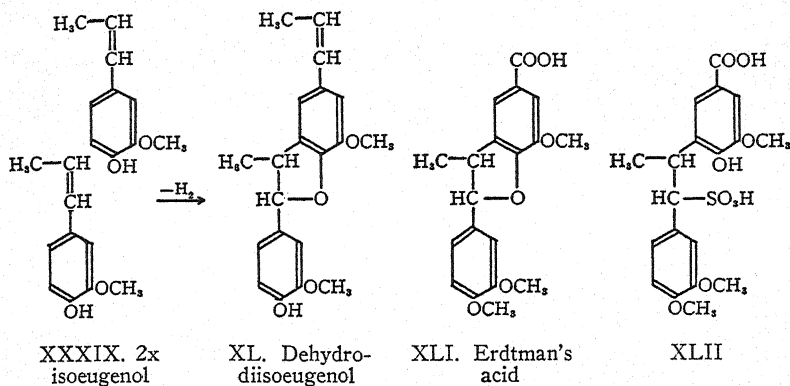
In the same manner the blue solution of potassium in ammonia at  $20^\circ$  splits off a part of the methyl groups. Ether bonds and oxygen-containing rings are simultaneously ruptured. Subsequent to methylation and oxidation 5 per cent veratric acid, 1.5 per cent isohemipinic acid, and 0.5 per cent dehydrodiveratric acid were recovered. The last-mentioned compound probably resulted from the action of atmospheric oxygen previous to the methylation.

The fact that all of the degradation methods described on pp. 99 to 105 may be explained in the same manner demonstrates the applicability of this conception. The following experiments on substances used as models for these reactions constitute a further support of the theory.

#### DEHYDRODIISOEUGENOL AND ITS CONVERSION PRODUCTS AS MODELS

The synthesis of dehydrodiisoeugenol (XL) (96) from two molecules of isoeugenol (XXXIX) by means of biochemical dehydrogenation with fungi (97) or through gentle chemical oxidation (97) is of great interest to the lignin problem as here is a case of simultaneous condensation and ring closure.

<sup>8</sup> Care must be exercised in carrying out this reaction.



After methylation and oxidation, Erdtman's acid (XLI) is obtained—a substance which fulfills nearly all of the requirements for a lignin model (84).

Following treatment with alkali, methylation, and oxidation, Erdtman's acid yields 21 per cent of veratric acid (instead of 53 per cent) and 5 per cent isohemipinic acid (instead of 66 per cent) whereas lignin under these conditions yields 14 per cent veratric acid and 4 per cent isohemipinic acid. Thus the ring is ruptured by alkali.

Under the conditions of the sulfite treatment the acid builds a sulfonic acid (XLII) from which a crystalline derivative may be obtained (98). The sulfonic acid yields by methylation and oxidation 17 per cent veratric acid (instead of 39 per cent) and 4 per cent isohemipinic acid (instead of 40 per cent). The maximum yields from ligninsulfonic acid are 3 per cent of veratric and 3.8 per cent of isohemipinic acid.

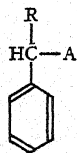
Erdtman's acid also reacts with thioglycolic acid. From the heterogeneous reaction product, by methylation and oxidation, 7.4 per cent of veratric acid (instead of 41 per cent) and 3 per cent isohemipinic acid (instead of 59 per cent) are obtained. Ligninthioglycolic acid yields 4 per cent and 3 per cent respectively.

It has recently been proved (33) that analogies between Erdtman's acid and lignin exist with reference to treatment with methylalcoholic hydrogen chloride (after methylation and oxidation 25 per cent veratric and 5 per cent isohemipinic acid are obtained) and with hydrazine.

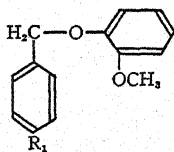
## OTHER MODELS

Bror Holmberg (99) has shown that phenylmethylcarbinol and diphenylcarbinol react with bisulfite, thioglycolic acid, or alcoholic mineral acids under the same conditions as lignin to form compounds of type XLIII ( $A = \text{SO}_3\text{H}, \text{SCH}_2 - \text{COOH}, \text{OC}_2\text{H}_5$ ). Furthermore the ethers of these carbinols react with thioglycolic acid in the presence of mineral acids. Benzylguaiacol (XLIV,  $R_1 = \text{H}$ ) does not react in the same manner as does lignin with bisulfite or with aqueous sulfur dioxide (both of which reagents hydrolyze the compound) or with thioglycolic acid (100). On the other hand anisylguaiacol ( $R_1 = \text{OCH}_3$ ) and the corresponding nitro compound ( $R_1 = \text{NO}_2$ ) react with bisulfite or aqueous sulfur dioxide to form anisylsulfonic (or nitrobenzylsulfonic acid) and guaiacol. Thioglycolic acid does not react. The guaiacol ether of methylphenylcarbinol (XLV) is completely analogous to lignin with reference to its reaction with bisulfite, aqueous sulfur dioxide, and thioglycolic acid.

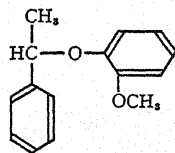
The experiments on models eliminate all doubt as to the type of the most important degradation reactions of lignin.



XLIII.  $R = \text{CH}_2$  or  $\text{C}_6\text{H}_5$ .  
 $A = \text{SO}_3\text{H}, \text{SCH}_2 \cdot \text{COOH},$   
 $\text{OC}_2\text{H}_5$



XLIV



XLV

## CONIFERYL ALCOHOL AS A MODEL

The monomeric crystalline form of coniferyl alcohol (VII) which is separated from its glucoside, coniferin, by the action of emulsin, is slightly soluble in cold water. A trace of mineral acid suffices to precipitate it as an amorphous polymeric substance which in regard to its methoxy content as well as its behaviour following methylation and subsequent oxidation (forming 22 per cent veratric acid, no isohemipinic acid) does not differ from the monomeric compound. Through energetic treatment with acid the polymeric form loses water. It is soluble in alkalis. Upon being boiled with alkali in the same way as lignin, methylated, and oxidized, it yields 26 per cent veratric acid, but no isohemipinic acid (101). The water-free polymeric form is

for the most part soluble in bisulfite at 135°. From the reaction mixture, 5 per cent vanillin can be recovered. By methylation and oxidation, no degradation products were obtained except a small amount of veratric acid. Thioglycolic acid also reacts with coniferyl alcohol, but, likewise, the addition compound after methylation and oxidation yields only a small amount of veratric acid.

The guaiacol group does not appear to have taken part in any of these reactions. Polymerization and subsequent loss of water occur in the side chain. It is probable that these reactions are caused by double bonds, part of which may be in conjugation with the ring. If crystallized coniferyl alcohol is treated with ferric chloride in alcoholic solution, warmed with alkali (corresponding to lignin decomposition), methylated and oxidized, then approximately 1 per cent of isohemipinic acid is obtained in addition to 8 per cent of veratric acid. The process is thus similar to the synthesis of dehydrodiisoeugenol. Hence it is natural to regard lignin as a dehydrogenation product of coniferyl alcohol (101).

#### ORIGIN OF LIGNIN

In this paper an attempt has been made to explain the chemistry of lignin on the assumption that as a natural product it is built according to a definite pattern. According to this pattern, one group is linked to other similar units, and this can be repeated without limit. This principle can be recognized in every high molecular natural substance (102), and it is improbable that lignin should constitute the sole exception. The fact that lignin chemistry can, with the assistance of this conception, and only with it, be coherently explained proves that the idea must in general be correct. It must be emphasized that the units belong to a small group of biochemically related types. This applies to the substances of formulae I to IV as well as the syringic component, VI, and to every unit which contains the methylenedioxy group. Such relationships are frequent. It is difficult to find a more homogeneous, biochemically intelligible linkage principle. The oxidative condensation of isoeugenol, which can be repeated with coniferyl alcohol, suggests the form of reaction by which the majority of guaiacol groups can become united. It is not necessary, or even probable, that the sequence portrayed on p. 94 *et seq.*, *i.e.*, etherification, then condensation, actually applies. It is more probable that both processes occur together as is the case with isoeugenol.

The problem of establishing the chemical constitution of lignin consists in distinguishing the various structural units of which it is composed and determining how they are linked together.

A survey shows that lignin enters into four distinct types of chemical reactions:

The first type of reaction concerns the functional groups: the hydroxyl, methoxyl, and methylenedioxy groups, the C-methyl groups and their derivatives. The second type is concerned with the substitution reactions, *i.e.*, the reactions with chlorine, bromine, nitric acid, mercuric acetate, etc. The third type consists of degradation without rupture of carbon to carbon linkages, *i.e.*, hydrolysis with alkalis, aminolysis, sulfite reaction, alcoholysis, etc. These are reactions in which the carbon frame is not attacked. It has been shown that all of these reactions can be explained as reactions of the  $\alpha$ -phenylcarbinols and of the open and the cyclic ethers. The most important reaction of lignin is the splitting of ether linkages (103). This is the principal reaction in lignin chemistry.

The last type of reaction consists of the degradation of the carbon framework of either lignin or of its conversion products. Degradation results from fusion with potash, oxidation, hydrogenation, or thermal destruction.

The origin of lignin remains unexplained. It is self-evident that ultimately it is derived from carbohydrates. It must be assumed that cellulose plays no rôle. If pectin should yield the starting material, as is frequently maintained (104, 105), then it must first be decomposed to uronic acid. If one is to synthesize from the latter a skeleton containing nine carbon atoms, the question arises whether or not the carboxyl group takes part in building the frame. This is exceedingly improbable. It is more likely that every conversion of uronic acid results in a pentose. However, it is difficult to conceive of the lignin skeleton being constructed from pentoses. It is simpler to assume that a hexose condenses either with a  $C_3$ -compound, corresponding in oxygen content to glycerine, or with formaldehyde and a  $C_2$ -compound corresponding to glycol.

The fact that the pectin in young tissues disappears as these tissues are transformed into wood does not prove that pectin is converted into lignin. The pectin may be changed into other substances during this active cellular transformation. Although it is generally agreed that pectin disappears, it is possible that it is only masked by other substances or that it is combined in such a way as to be undetectable

(106). Norman (38), who has contributed the best critical survey of this subject, has pointed out that the amount of pectin found in young tissues is insufficient to build all of the lignin which is later found in the wood.

If lignin does not come from pectin then it may be surmised that it comes from hexoses. An approach to the understanding of the synthesis of lignin in nature will be made, however, only when the origin of the phenols, especially those of the  $C_6$ -group, is known.

Vanilloyl-methyl-carbinol [ $1 - CH_3CHOH \cdot CO - 3 - MeO - 4 - OH \cdot C_6H_5$ ] (XXXVa), corresponding to 4 per cent of the lignin content, has been detected by H. Hibbert (107) in the low molecular portion of spruce lignin. This unit harmonizes with our representation whether it occurs as such in the lignin portion of wood or is formed through rearrangement of units III-IV. Inasmuch as lignin upon treatment with chromic acid yields but 6 per cent acetic acid [(= 2.7 per cent  $C - CH_3$ ) page 93] only a part of the lignin (18 per cent at the most) could result from this unit. Approximately 33 per cent of the methoxyl groups present in spruce wood are, subsequent to suitable oxidation, isolated in the form of vanillin (page 98) (82) which likewise can not directly result from component XXXVa or its condensation products. The piperonyl component accounts for 25 per cent and the syringyl component for approximately 5 per cent of spruce lignin (page 96). The substance XXXVa is a derivative of benzoylmethylcarbinol. The majority of the lignin units are, however, derivatives of phenylethylcarbinols (I-III) as is shown by the reactions with sulphurous and thioglycolic acids, which up to the present time have been noted only on  $\alpha$ -phenylcarbinols and their derivatives.

At last it seems agreed that lignin is a derivative of phenylpropane having three oxygen equivalents in the side chain and, furthermore, that it is present in wood in different degrees of condensation varying from simple units to complex aggregates. It is explicitly emphasized (4, 1) that schemes I-III as well as XII-XXIII are but examples of the many possibilities.



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# X-RAY STUDIES OF THE STRUCTURE OF COMPOUNDS OF BIOLOGICAL INTEREST

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Biology is fast becoming a molecular science, a desire to tread as far as possible the friendly ground of physics and chemistry and see where it leads. It may be that the angels are right, but it is good to feel and take part in a foolishness that is the scientific hall-mark of our times. The search is now for the structure and arrangement of the molecules of living things.

Chief among these molecules are the proteins, and the greatest excitement these days is about the proteins. Theirs, then, must be the chief place in this review.

## THE PROTEINS

It is a biochemical commonplace that proteins are polypeptide chain systems—such a commonplace as to be possibly untrue! Most probably it is true, but there is a feeling around of “not proven.” Certainly the fibrous proteins are polypeptides, and the “globular” proteins yield polypeptides on denaturation; but what of the globular proteins before they denature?

The polypeptide chains in proteins first became “real” in the light of the x-rays: Meyer & Mark (1) showed that the x-ray photographs (2) and properties of silk follow very simply if the fibre is thought of as a kind of molecular yarn made from chain molecules, fully-extended, orthodox polypeptides. But the argument held only in part for other protein fibres, such as hair and muscle: both the elastic properties and the repetition of pattern along the fibre axis are quite different from those of silk. There is no need to go into these things again here, but the solution given by x-rays is that the polypeptide chains are just as real in hair and muscle, but they are not normally fully extended (3). They may be pulled out straight by stretching the wetted fibres, but they return to their regularly-folded equilibrium form on letting go. This is the basis of their long-range elasticity, an intramolecular phenomenon of deep biological importance.

The question has arisen from time to time whether the x-ray photographs of hair and similar keratin structures really come from the true elastic substance or from some secondary histological component.

Accumulated indirect evidence has to all intents and purposes long negated the second possibility, but recently the matter has been set at rest by the work of Woods (4) on the biological cells themselves. The cells of hair, wool, horn, etc., may be separated largely intact by controlled retting with trypsin or certain bacteria, but even so it has not yet been found possible to obtain good x-ray photographs of single keratin cells because of their small size. Woods overcame this difficulty by the simple method—a method that should find other applications in biology—of orienting the cells electrically between two wires immersed in a suspension in water spread on a glass slide, and letting the suspension dry with the field kept on (5). By this means he formed thin tissues of cells all pointing the same way, and the x-ray photographs of these, of course, are effectively those of a single cell. In all cases the photographs obtained were of the familiar  $\alpha$ -keratin type, such as are given by the original keratin structures themselves.

By methods which need not be described here Woods obtained also the  $\beta$ -keratin photograph from stretched cells, made a study of the principal elastic properties of the cells in various states, and finally drew the general conclusion that, except for secondary differences, the x-ray and elastic properties of keratin in bulk are undoubtedly those of the actual cell substance.

Similar conclusions hold too for the cells of muscle—and we mean here something more than a formal resemblance between keratinous and muscular tissue. Boehm & Weber (6) first showed that the x-ray photograph of muscle is essentially that of oriented myosin, the chief solid component of the cells, and Weber (7) has made extensive optical and similar studies of myosin in relation to muscle; but the point of view that is fundamental seems to turn on this, that the x-ray and elastic properties of myosin are closely related to those of keratin. It was noticed several years ago (8) that hair and muscle photographs are almost unbelievably alike, and this observation has now been developed into a detailed comparison, taking in all the main types of x-ray photograph and changes of state of mammalian hair, living and dead muscle, and pure isolated myosin (9). Only preliminary reports (10, 11) have appeared as yet, but pending the fuller discussion (9) it is possible to summarise the results quite briefly. From several lines of evidence there can be little doubt that myosin is the active elastic principle in muscle, and x-rays show that it is present as a swollen solid, a fibrous protein with the polypeptide chains running along the length of the fibrils. The chains in relaxed muscle are not fully ex-

tended, as was originally thought, but are regularly folded in a configuration like that of  $\alpha$ -keratin, the protein of unstretched mammalian hair, etc. Like keratin again, myosin can be stretched into the fully-extended, or  $\beta$ -, configuration and is elastic over the same long range, and—what is most important of all—it shares with keratin the power of contracting to a length much shorter even than that of the  $\alpha$ -form. Untreated keratin has not this power of “supercontraction,”<sup>1</sup> but only keratin that has suffered a certain breakdown in the side-chain bridges between neighboring main-chains; and the true analogy is not between myosin and normal keratin, but between myosin and the labile, or “supercontracting,” form of keratin. With this proviso the analogy is remarkably complete—the main dimensions of the polypeptide grids are almost identical, and all the principal x-ray and elastic transformations that had been observed with supercontracting keratin have been duplicated with myosin. The contraction of muscle, then, corresponds to the supercontraction of hair, and the changes in the x-ray photographs of muscle during contraction may be described most simply by saying that they are similar to what is observed when hair supercontracts. There is little disorientation indeed of the  $\alpha$ -pattern in the first stages of contraction; and in fact the disorientation at all stages is smaller than what might be expected from the accompanying contraction: disorientation alone of structural elements does not explain the elastic behaviour of muscle either quantitatively or in principle, and the most reasonable interpretation from all the known properties of myosin and keratin, and from studies of both plain and striped muscle, is that muscle contraction is the expression of a further intramolecular folding of the already folded polypeptide chain system.

It will be seen that in this study the muscle problem is approached from a new angle, and ultimately the argument derives its strength not so much from what we know about muscle itself as from what we know about keratin: all questions of activation are set aside for the moment, and we show that there is another system, that of hair, that is free from these complications yet still has the essential elastic properties of muscle. And since the isolated muscle protein myosin is so closely related to the hair protein keratin, the problem resolves itself into that of the common molecular structure of myosin and keratin.

In a sense this demonstration of the affinity between myosin and

<sup>1</sup> So called to distinguish it from the more familiar recovery from the  $\beta$ - to the  $\alpha$ -form.

keratin relegates the muscle problem rather to a place of secondary importance, or at least makes of it only a special case in something that goes far deeper and wider; for the common molecular configuration of myosin and keratin is not confined to these two proteins alone; it is shared also by the fibrous proteins of the epidermis (12). Indeed, x-rays show that if we take into consideration its  $\beta$ -subgroup, the myosin-keratin group includes all epidermal structures and muscular tissue,<sup>2</sup> and that outside these, most or all other native protein fibres fall into the collagen group (11). Probably only these two basic groups suffice to describe the whole of the fibrous proteins, and there are even indications in the relations between connective tissue and muscle that perhaps in the end there will be found to be only one.

The collagen group comprises connective tissue, tendons, cartilage, elastoidin (13, 14), such structures as jelly-fish (15) and the threads of the sea cucumber (16), etc. The x-ray pattern is quite different from that of the fully-extended  $\beta$ -proteins, yet it seems fairly certain that the polypeptide chain system is stereo-chemically fully-extended—perhaps in a cis-configuration (17), as opposed to the trans-configuration of the  $\beta$ -proteins. Above certain specific temperatures the collagen fibres contract rapidly (18), sometimes to about one quarter of their original length, and while in this state they show long-range elasticity like that of keratin and myosin. The range of contraction and the x-ray photographs (13, 14) suggest something analogous to the supercontraction of keratin and myosin—in any case, there can hardly be any doubt that a folding of the polypeptide chains takes place. Elastin, from the ligamentum nuchae for instance, shows striking long-range elasticity even at ordinary temperatures and gives the typical collagen pattern only on stretching (19), so it must be classified as a member of the collagen group with an abnormally low thermal transformation temperature (11).

Figures 1 and 2 illustrate the four main types of x-ray fibre photograph and corresponding suggested configurations for the polypeptide chains (20). The x-ray pattern of feather keratin, which is given also by reptilian scales, etc., is richer than any of those shown, but in reality it is only an elaboration of a slightly contracted  $\beta$ -type (21).<sup>3</sup>

<sup>2</sup> The existence of myo-epithelial tissue, that is both skin and muscle, follows almost as a direct deduction from the x-ray data (9).

<sup>3</sup> Oyster silk (22) is an elastic protein fibre that gives normally an x-ray powder photograph that is difficult to classify for the moment.

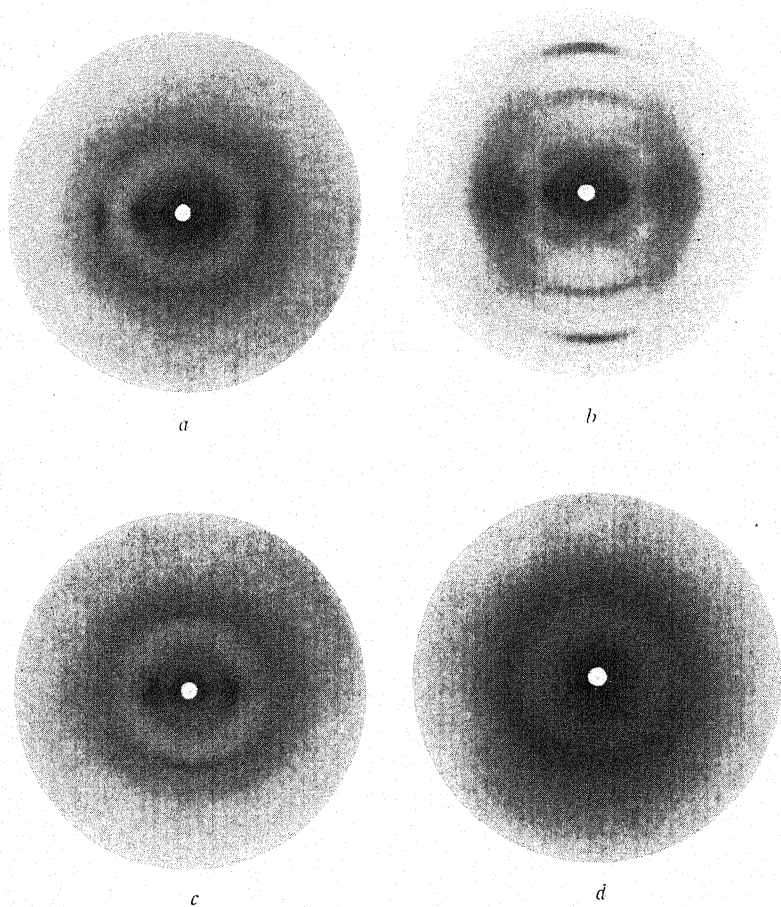
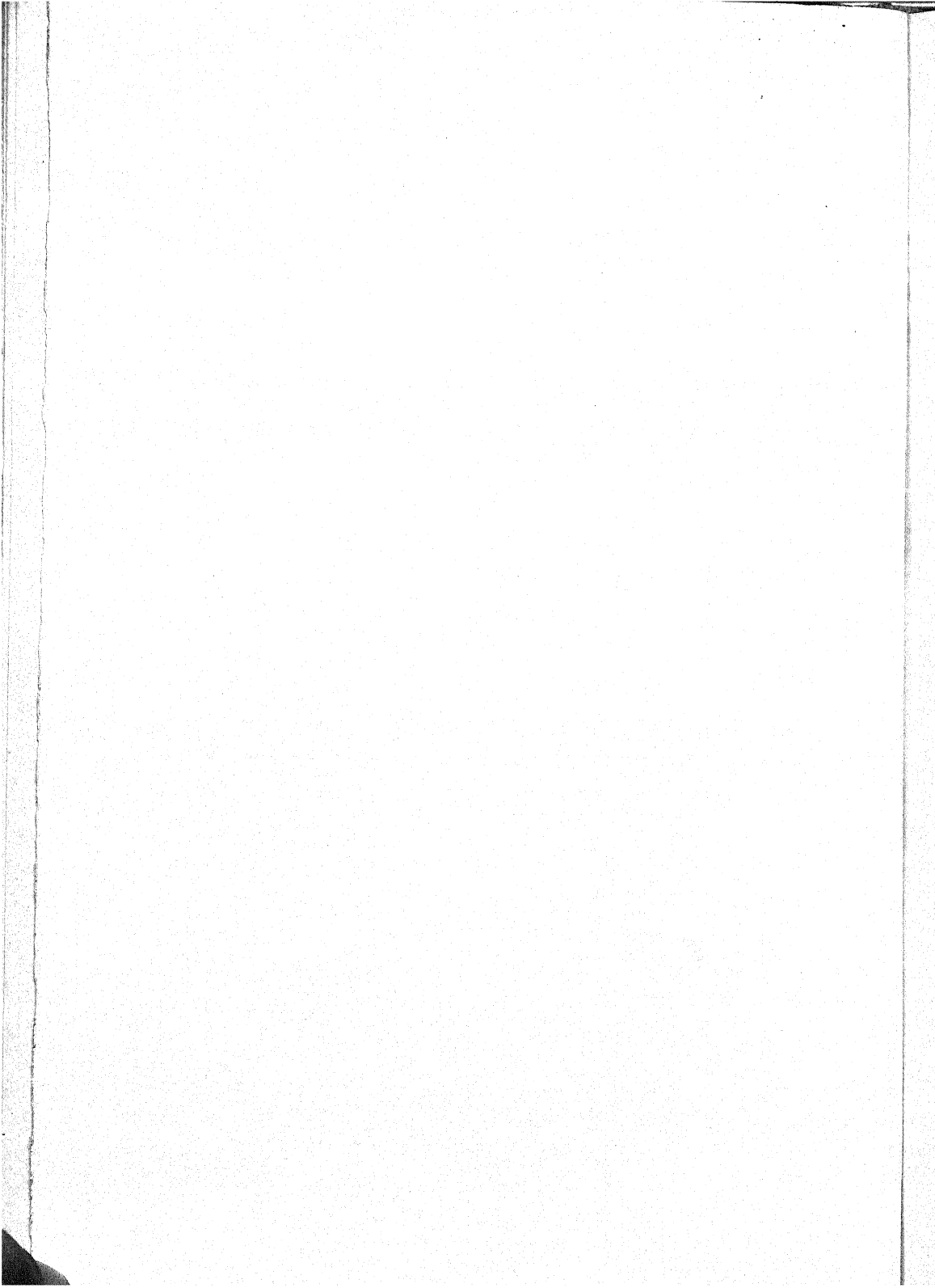


FIG. 1.—The four principal types of x-ray photograph given by fibrous proteins: (a)  $\beta$ -type (stretched myosin); (b) normal collagen type (tendon); (c)  $\alpha$ -type (unstretched myosin); (d) "supercontracted" collagen type (elastoidin contracted in hot water).





The x-ray interpretation of the structure of the elastic fibrous proteins surely points directly to the solution of the problem of the "globular" proteins also: it is almost inconceivable in any case that there are two distinct problems, and once having demonstrated the principle of intramolecular folding, if only along one direction, the natural conclusion is that the massive roundish molecules of the globular proteins are but generalised examples of the same principle (23). That they finally give rise to polypeptide chains on denaturation

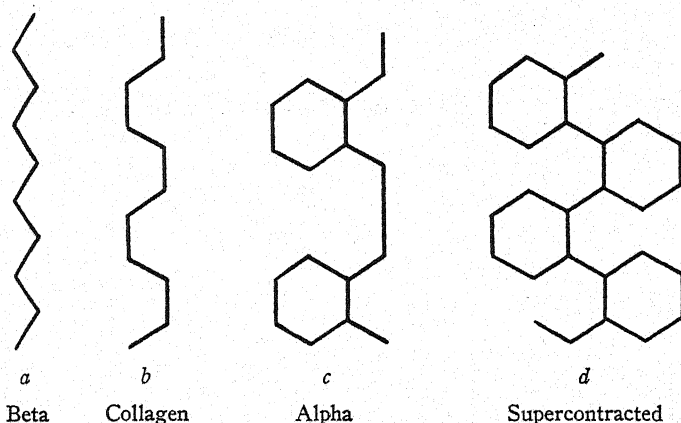


FIG. 2.—Suggested scheme for the four principal states of fibrous proteins (cf. Fig. 1)

appeared from the fact that all denatured proteins yield an x-ray photograph of the disoriented  $\beta$ -type (24), but the argument was clinched when it was shown that solutions in urea of the seed globulins, for instance, can be spun into elastic fibres which give on stretching an x-ray photograph like that of oriented  $\beta$ -keratin (25). Here, undoubtedly, we have long polypeptide chains produced on denaturation, chains that can actually be drawn out to form synthetic fibres analogous in structure to the natural fibres—yet still it is not proved that the chains pre-exist as such in the original globular molecules. Chemists themselves are wondering whether enzyme studies really do point to peptide linkages in the undisturbed molecules (26), and there are at least possibilities of fabrics held together in other ways (27), or of a transformation such as ring-chain polymerisation (28).

In spite of these objections, however, the evidence seems still in favour of a rather simple unfolding process for most cases; other-

wise, how explain the reversible transformation from  $\alpha$ -keratin and myosin to  $\beta$ -keratin and myosin just by stretching in the presence of water? Specially does the x-ray evidence indicate that the transition from the native to the denatured state is easy, and though there may not be always *long* chains present in the globular proteins, it is not difficult to conceive of a mechanism of fibre formation by interchange of peptide linkages between shorter folded groups (25, 29). In general, the folds will be stabilized and smaller units linked together by side-chain bridges, and we should expect the activation of sulphhydryl groups, for instance, on disintegration of the specific folded configuration. Denaturation does not necessarily involve side-chain breakdown, though: it may involve side-chain combination in the case of a fibrous protein such as myosin, which may be said to be already "configurationally disposed towards denaturation" (25). X-rays show that myosin denatures in two steps, first (on drying) by aggregation of the  $\alpha$ -form, and then (on heating) by unfolding and aggregating as the  $\beta$ -form (9). It is only during the latter step that we should expect activation of sulphhydryl groups, and that is what is found (30). The easy transition of myosin from the  $\alpha$ - to the  $\beta$ -form on heating to no more than about 50° C, or sometimes only on standing at ordinary temperatures (9), is illuminating: we obtain a disoriented  $\beta$ -photograph just as if we had heated a "genuine" globular protein like egg albumin—yet we can produce  $\beta$ -myosin simply by pulling  $\alpha$ -myosin, and it will go back again to  $\alpha$ -myosin when we let go! Heat denaturation here is surely a question of unfolding.

The latest x-ray information on the unfolding of globular proteins comes from a study of protein multilayers (31), as built up on a metal slide by the technique of Langmuir & Blodgett (32). Films prepared by means of a specially constructed automatic apparatus, and consisting of up to 1764 monolayers of egg albumin, were detached from their metal base and examined directly. Optical and mechanical examination revealed fibrous properties parallel to the direction of movement of the slide through the liquid substrate, and these properties were shown by x-rays to arise from polypeptide chains oriented approximately in that direction, with their side-chains approximately perpendicular to the surface. The side-chain spacing was about 9.5 Å, as is found in protein fibres [the weight per unit area of protein monolayers was, in fact, deduced from x-ray fibre data several years ago (33)]. The thickness of the multilayers was then measured by means of an optical system which did not involve the optical properties of

the films themselves, and again the thickness per monolayer was found to be about 9.5 Å. Finally, the same order of thickness was obtained simply by folding the films repeatedly and measuring them with a screw micrometer.

Apart from the satisfaction of measuring for the first time the thickness of a polypeptide chain by such convincingly inelegant means, perhaps the most important conclusion to be drawn from these experiments is that an egg albumin monolayer consists of polypeptide chains sufficiently long and free to be oriented—to be “spun” as a polypeptide ribbon, so to speak—merely by pushing or pulling a flat slide through it. The polypeptide chains are preformed in the monolayer under the influence of surface forces (and almost certainly by direct unfolding), for if they were formed by denaturation after deposition on the metal slide, experiment shows that they would be completely disoriented.

Stenhagen (34) has now obtained x-ray reflections of spacing 9.5 Å from forty layers of lipoprotein without first stripping them from the metal base. He used the condensing monochromator described by Fankuchen (35), which constitutes one of the more outstanding recent technical developments in the x-ray study of compounds of high molecular weight (see below).

And so we find ourselves knocking urgently at the crystallographic door of the globular proteins to try to find out the secret of their folds. That way of approach has long been hindered by the smallness of the crystals and their instability outside their mother liquor—without special precautions only denaturation photographs are obtained (24)—but now there is progress.

For the present, to be sure, the x-ray conclusions on macroscopic protein crystals hardly amount to more than what we know from other sources, in the sense that they only confirm molecular dimensions given already by ultracentrifugal (36) and other physico-chemical studies; but further data and fuller interpretation are to be expected. Crystallographic cell dimensions, number of molecules per cell, molecular weight, and in some cases space-group also, have so far been worked out for the following: insulin (37), excelsin (25, 38), lactoglobulin (39), chymotrypsin (40), haemoglobin (40), pepsin (41), and tobacco seed globulin (42); while x-ray powder data are available for squash seed globulin (24), chymotrypsinogen (43), and Bence-Jones protein (44). Insulin has also been submitted to a Patterson analysis, but the interpretation is still under debate. Wrinch

(45) has proposed a cage-like structure for the molecule, based on the repeated folding of a polypeptide chain through the intermediary of lactam linkages such as had been postulated for the folds of  $\alpha$ -keratin (46), and she argues that this structure is the correct solution of the Patterson diagram (47). The outcome of the discussion will be awaited with considerable interest, for undoubtedly it contains the seed of further x-ray developments.

In the meantime it is pertinent to look at the matter from a more general point of view (48). One of the most intriguing features of Svedberg's tabulated data (36) is the wide variation of molecular shape within one and the same molecular weight group—the 35,000 group, for instance, which includes insulin. Insulin, it is true, may be effectively nearly round, but there are other members of the group, notably zein and erythrocrucorin from *Chironomus*, that must be far from round. Yet most people feel now that Svedberg's molecular weight groups are really structural or configurational groups: the molecular weights within any one group are approximately the same because each member is presumably built from the same number of amino acid residues. The most important property of Wrinch's cyclol structures, of which  $C_2$  has been proposed for insulin and pepsin (49), is that they are indeed configurational groups whose molecular weights fall into an approximate quantum series like Svedberg's, and what is more, the  $C_2$  cyclol is constructed from 288 residues, which would satisfy the 35,000 group and fit in with the latest chemical views (50)—but the number 288 is the outcome of a certain size and shape, of a definite kind of linkage on the surface of a truncated tetrahedron; and though size and shape may agree well enough for insulin and pepsin, how are we to extrapolate to other shapes of similar molecular weight? We might use various arrays of  $C_1$  cyclols, each of 72 residues, but then the density would be different; and anyway, we should still be only on the fringe of the problem, for it seems to be the fact that similar molecular weights may be associated with degrees of extension up to that of a fully-extended polypeptide chain! The argument is as follows.

From new analytical data supplemented by the results of other workers, Bergmann & Niemann (50) conclude that both the total number of amino acid residues in a protein molecule and also the numbers of each of the various residues are expressible in the form  $2^n 3^m$ ; and on this basis the minimum molecular weights of chicken egg albumin, cattle haemoglobin, cattle fibrin, and silk fibroin are

found to correspond to  $288^4$ ,  $2 \times 288$ ,  $2 \times 288$ , and  $9 \times 288$  residues, respectively. The point now that strikes the x-ray analyst (52) about these findings is not simply that they provide an experimental stoichiometrical basis for the Svedberg generalisation of multiple molecular weights, but that the Svedberg generalisation, derived from the study of soluble globular proteins, appears to hold also for undoubted fibrous proteins; for the verdict of x-rays is that both fibrin (53) and fibroin (1, 2) are fully-extended,  $\beta$ -proteins.

A similar inference may be drawn from x-ray and density data on  $\beta$ -keratin (52, 48)—and in this connection it should be noted that such data provide a direct measure of the true average residue-weight, a quantity that can only be surmised from incomplete chemical analyses. The average residue dimensions in  $\beta$ -keratin are  $9.7 \times 4.65 \times 3.33$  Å, and the density is 1.3, so that the average residue-weight is  $(9.7 \times 4.65 \times 3.33 \times 1.3) / 1.65$ , or about 118, corresponding to approximately 0.85 gram-residues per 100 gm. of keratin. According to the Bergmann-Niemann theory, the number 0.85, when divided by values of  $2^{n3m}$ , should give the numbers of gram-residues of the various acids in 100 gm. of keratin. Table I

TABLE I  
DISTRIBUTION OF AMINO ACID RESIDUES IN WOOL KERATIN

Acid	Frequency (F)	Gm.-res. in 100 gm. wool	
		Calc. (0.85/F)	Obs.
Glutamic .....	8 ( $2^3$ )	0.106	0.103
Arginine .....	16 ( $2^4$ )	0.053	0.059
Aspartic .....	16 ( $2^4$ )	0.053	0.054
Tyrosine .....	32 ( $2^5$ )	0.027	0.027
Lysine .....	48 ( $2^4.3$ )	0.018	0.019
Tryptophane .....	96 ( $2^5.3$ )	0.008 <sub>9</sub>	0.009
Histidine .....	192 ( $2^6.3$ )	0.004 <sub>6</sub>	0.004
Amide-N .....	9 ( $2^2$ )	0.094	0.098
Cystine* .....	8 ( $2^3$ )	0.106	Mean value
Methionine .....	192 ( $2^6.3$ )	0.004 <sub>6</sub>	Mean value

\* Calculated as half-cystine residues.

shows these numbers compared with the most reliable chemical data. If we disregard the amide nitrogen and the sulphur acids, the keratin

<sup>4</sup> It has been pointed out by Taylor (51) that the published figures for egg albumin lead not to 288, but to 72 residues.

molecule must contain a minimum of 192 residues; but if the number of amide groups is also a submultiple of the total number of residues, as is not unlikely, then the molecule must contain a minimum of  $2 \times 288$  residues (molecular weight about 68,000, like haemoglobin, for instance). In any case, it will be agreed that the results are distinctly in favour of the view that keratin, too, will ultimately be found to conform to the common stoichiometrical plan.

It is still too soon to be quite sure about the detailed correctness of the Bergmann-Niemann theory, but the basic idea must be right. There may be a kind of amino acid replacement, a sort of "intramolecular isomorphism," but there cannot be anything haphazard about the structure of the proteins, and 2's and 3's certainly are there in the analytical ratios of the amino acid residues. And in the x-ray photographs, at the very least, regularity of structure and persistence of pattern are plain for all to see: the germ of the Bergmann-Niemann theory, in fact, lies in the x-ray study of gelatin (51).

However, the point we are aiming at is this, that there is apparently a common factor in the synthesis of all proteins, whether fibrous or globular, and their shapes and sizes, as we know them now, seem to be somehow of a secondary consideration. The original distinction between fibrous and globular has almost disappeared. The units of a "globular" protein<sup>5</sup> may be long, as are those of the tobacco mosaic virus, and the rod-like units of the fibrous proteins are in reality comparatively thick, not single chains, but at least "polypeptide grids," to judge by the large side-spacings that have now been discovered (55). Feather keratin (21) is a splendid example of a protein with a foot in either camp: its fibrous and polypeptide nature are beyond dispute, yet it has a giant intramolecular pattern.

The combined impression we receive from the different lines of approach is that all proteins are fundamentally of a fibrous structure,<sup>6</sup> built up by the successive aggregation of relatively small units. And the Svedberg series and the results of Bergmann & Niemann suggest

<sup>5</sup> Perhaps a better term, proposed by Ostwald (54), would be "corpuscular" protein.

<sup>6</sup> All proteins have the same order of density, for instance, that which may be inferred from x-ray data on the fibrous proteins (33). As an impressive example of this, Dr. H. B. Bull has kindly informed me of the following densities of egg albumin measured in hydrogen at 25° C: native,  $1.350 \pm 0.008$ ; surface denatured (coagulated),  $1.339 \pm 0.005$ ; heat denatured (coagulated),  $1.347 \pm 0.004$ .

forcibly, too, that Nature uses only a few basic configurations out of the enormous number of theoretical possibilities. Amino acid constitution also is not the chief consideration, and the situation seems to be something like that in the field of aromatic compounds, all of which may be derived from the benzene ring and its homologues. Empirical analyses of such compounds would be utterly confusing, were it not for the guiding principle of the hexagon. Some such limitation must be at the heart of the proteins, too.

The principle of constant structure with varying constitution does not hold merely for the orthodox globular proteins. One of the most surprising things brought to light by x-ray studies of the fibrous proteins is the fact that there are probably only two main structural types; and *within each of these two types there are again wide variations of constitution such as are found between members of each of the Svedberg groups* (11, 9). So on still one more ground, and perhaps the most important of all, we arrive at a conception of unity, unity amongst all proteins. They are difficult in details, but not in principles.

#### NUCLEIC ACIDS AND NUCLEOPROTEINS

What are these principles? We do not know yet, except in the most general terms; but obviously we are getting nearer. Something like a configurational crisis is at hand even now.

But we are still too short of data on many vital points, and this tends to narrow our outlook, to restrict our interpretative powers. The problem of protein synthesis is not one of proteins alone, but of proteins plus other molecules—saccharides, nucleic acids, etc. This ghost of a generalisation that is looming up, that different amino acid constitutions may be associated with similar structures, hints at a world behind it, and activities of which we are unaware. When proteins are born, other molecules assist at their birth; and perhaps chief among them are the nucleic acids. The earliest reproductive processes that we know, those of the viruses and the chromosomes, always involve protein and nucleic acid.

The nucleic acid present in the chromosomes and certain glandular tissues is thymonucleic acid. Its sodium salt forms viscous solutions from which oriented preparations may be made that give excellent x-ray fibre photographs (56, 48). The strongest period along the fibre axis is at  $3.34 \text{ \AA}$ , and this key observation, taken in consideration with the high density, the fibrous properties of the solid state, and the



properties of the solutions with their optically negative streaming double refraction (57), indicates that the structure of thymonucleic acid is that of a column of nucleotides piled on top of one another. In the preparations in question there must be of the order of 2000 nucleotides per column, and the true intramolecular pattern along the column repeats at a distance equivalent to the thickness of 17 nucleotides at least—but what this means in terms of the chemical finding that thymonucleic acid is a *tetranucleotide* is not clear at the moment. The really interesting point, though, is that the spacing of 3.34 Å between successive nucleotides is almost exactly equal to the distance between successive side-chains in a full-extended polypeptide (52, 56), which can hardly be an accidental agreement, in view of the nucleic acid changes that occur during mitosis (58). The idea takes shape that at some stage in the chromosome cycle, involving elongation of the polypeptide chains, the conditions may be defined by just this correspondence.

Each nucleotide in a nucleic acid is believed to be linked to its neighbours through phosphoric acid (59), and so since there is such a close match between nucleotide period and side-chain period, combination with the sperm protamine, clupein, should be easy: Linderstrøm-Lang & Rasmussen (60) have shown that clupein is a simple polypeptide of about 28 residues, 21 of which are arginine residues, and we should expect clupein thymonucleate to be fibrous like the sodium compound, with a similar dominating intramolecular period of about 3.3 Å. This has turned out to be so (48), and it is striking indeed how much the x-ray photographs of the two derivatives resemble each other. Presumably the polypeptide chain just attaches itself down the side of the nucleic acid column, leaving the stereochemical frameworks of both more or less unchanged.

The fibres of clupein thymonucleate are optically negative with respect to their length (48), just as are the nucleic acid-containing (58) bands of the salivary chromosomes with respect to the chromosome axis (61); which means that if the protein-protamine compounds in the chromosomes are of a similar nature, then the protein chains must run along the length of the chromosomes. Thus for the first time we establish experimentally a possible molecular basis for the physico-chemical properties of the chromosomes, their linear genic pattern, and the phenomena of breakage, reunion, translocation, etc., that may accompany mutation; for nothing seems more reasonable at the present state of our knowledge than that life is inseparable from

the proteins, and different side-chain sequences, perhaps modulated by combination with nucleic acid, might well be the first themes of organic development.

To the molecular biologist there can be no question but that the most thrilling discovery of the century is that of the nature of the tobacco mosaic virus (62): it is but a nucleoprotein. A dozen or so other viruses have now been isolated—different strains from tobacco, cucumber, potato, tomato, etc. (63)—and they are all nucleoproteins, “straight” compounds that give x-ray crystal photographs. X-ray examinations have been carried out by Wyckoff (64) and Bernal (65) and their collaborators, but mostly by the latter group, who have submitted three strains of tobacco virus (mosaic, enation and aucuba) and two of cucumber to an intensive study. The units in pure preparations of these are similar, apparently rod-like bodies,<sup>7</sup> about 150 Å thick when dry and at least ten times as long as they are thick. All the preparations, except the weakest solutions, are actually liquid-crystalline: the rods fit together laterally in two-dimensional hexagonal close packing, but have no regularity of packing in the direction of their length. And the fascinating thing is this, that the x-ray pattern is composite, one part being a fibre diagram that remains practically unchanged in all preparations from solutions up to the dry gel, and the other a system of side reflections whose spacings vary with the proportion of water present. The former is the *intramolecular* pattern—the units are of such great size and internal regularity of structure that, like the diamond, we may think of them as molecules or as crystals—while the latter is an *intermolecular* pattern.

The intramolecular pattern is of a similar order of complexity to that of feather keratin (21), and it repeats along the fibre axis at about 69 Å. It is telling us, of course, that the giant molecules are built up in quasi-crystalline fashion, from subunits that are relatively small. Bernal (68) now favours a subunit of dimensions about  $11 \times 10 \times 10$  Å, but he originally suggested  $22 \times 20 \times 20$  Å (65). It can be shown (69) from the nucleic acid content (5 per cent) and x-ray data on the proteins and nucleic acids (56, 48) that the larger dimensions correspond at any rate to the smallest possible chemical

<sup>7</sup> There is still some disagreement (66) as to whether the true unit, as it exists in the diseased tissues, is really rod-shaped: there is evidence that it may be almost round like that of the tomato “bushy stunt” virus (67), but is aggregated into rods by the methods of isolation.

subunit, for the volume they include is associated with just one nucleotide—in combination with some 54 amino acid residues.

The intermolecular pattern, as already indicated, arises from a two-dimensional hexagonal close packing of the virus rods with respect to one another. Their lateral separation is increased to about 210 Å in the wet gel (as opposed to 150 Å in the dry gel), and to as much as 450 Å in a 13 per cent solution. And all this, while maintaining uniform hexagonal regularity—a truly amazing state of affairs! But to adapt the words of a popular song, the tobacco mosaic virus is “an education in itself”: quite apart from its outstanding biological implications, it raises so many important points in colloid and diffraction theory, not to mention the stimulus it has given to the x-ray technique of measuring very high spacings (35), that it would require a separate review to do it justice.

But here we may only hint at such things, and sound the high note of the virus to conclude this account of our present x-ray knowledge of the proteins. It is a hopeful note, if not yet a triumphant note, for the boundaries are down between the living and nonliving, and we see “life” in perhaps its simplest possible manifestation, as an autocatalytic reproduction of single chemical units!

### POLYSACCHARIDES

Increasing activity during these last years in fields related to the proteins somewhat overshadows such developments as have occurred in our knowledge of cellulose and other polysaccharides, but from the biological viewpoint at least it is worth while considering once more how we stand with the latter. From the structural viewpoint the cellulose situation is not really very much clearer, in spite of further x-ray studies by Sauter (70) and Meyer & Misch (71), and the warm debate (72) the new data have occasioned.

With his improved technique (73) Sauter has observed over forty new reflections from cellulose and has deduced a cell of dimensions:  $a = 10.8$  Å,  $b = 10.4$  Å (fibre axis),  $c = 11.8$  Å, and  $\beta = 85^\circ$ . This cell contains eight  $C_6H_{10}O_5$  groups and is therefore twice the volume of the Meyer & Mark (74) cell ( $a = 8.35$  Å,  $b = 10.3$  Å,  $c = 7.9$  Å, and  $\beta = 84^\circ$ ) according to the latest measurements (71), besides being oriented diagonally with respect to it. As a matter of fact Sauter's cell, with slight modification, is no other than one already proposed by Sponsler (75).

The diagonal correspondence between the Meyer-Mark cell and the Sponser-Dore-Sauter cell was pointed out by Bragg (76), and there never has been any conflict with regard to the bare geometry: the difficulty concerns the relative orientations of neighbouring chains—which run one way along the fibre and which the other, or whether, indeed, they might not run all the same way—and the relation between successive glucose residues. Meyer's suggestion (77) that equal numbers of chains might run in opposite directions is statistically plausible at any rate for hydrate cellulose, but what happens in the synthesis and aggregation of native cellulose may be quite a different matter. Altogether, it cannot be said that the question of chain orientation is settled, and neither is it clear yet why successive glucose residues are crystallographically different. Perhaps the best explanation of this curious point is that given by Cox in a recent review (78). Cox's idea is that the strict dyad screw-axis along the chains is spoiled by an adjustment of hydroxyl bonds necessitated by a statistical distribution of reversed chains. Meyer's suggestion, it should be said, is of an ordered distribution.

It has been generally believed that native cellulose is only a metastable form of the polysaccharide, because it was not found possible, once it had been transformed into hydrate (mercerised) cellulose by treatment with strong swelling agents, ever to get it back again by direct means; but now that view has proved untenable: mercerisation has been reversed, and under conditions which indicate that after all native cellulose is the stable configuration over the temperature range investigated. Though ramie still remains in the native form after heating for five days in water at 150° C, partial conversion of hydrate cellulose into native cellulose takes place on heating for half an hour in water at 200° C, and almost complete conversion after half an hour in glycerine at 250° C (79). Native cellulose may also be recovered indirectly by way of the ammonia compound (80) or by way of alkali cellulose (81, 79).

However, this loss of stability status on the part of hydrate cellulose has been largely redeemed by its reasserting itself in another way, through the most interesting discovery of Sisson (82) that the native form of cellulose is *not* exclusively the so-called "native" form. The cellulose present in the single-celled marine plant, *Halicystis*—three species from different localities have been examined—turns out to be hydrate cellulose! This is the first example of its kind to be observed, opening up a new chapter in the chemistry of the

plant cell wall. A further important feature in the structure of *Halicystis* is that though the direction of the cellulose chains in the plane of the membrane is random, the (crystallographic) plane of spacing 7.4 Å tends to lie parallel to the surface. The plane of spacing 6.1 Å is similarly disposed in the green alga, *Valonia ventricosa*, for instance, but the cellulose there is in the native form (83), and the chains are confined to two sets making roughly an angle of 80° with each other (84).

The directions of the cellulose chains in *Valonia* have now been mapped out completely by x-ray methods (85)—again the first example of its kind, opening up a new chapter in the molecular topography of the plant cell wall. Each of the two directions departing from a chosen starting point was followed, photograph by photograph, all the way round a complete cell shaped roughly like a prolate spheroid. When the observations were plotted on a model, it was seen that the chain directions fell into two quite distinct sets, and a constant angle between the two was maintained because one set formed meridians to the spheroid, while the other formed what appeared to be logarithmic spirals closing down on its poles. It was as though we had a fibre whose cylindrical cell wall was composed not only of the usual helical spirals of cellulose chains but also of chains parallel to the fibre axis, and blew it up into the form of a prolate spheroid: the structure arrived at would be something like that of *Valonia*—but not exactly, because the wall of *Valonia* differs from that of the fibres in a remarkable feature: it consists of many thin laminae, and the chain direction alternates regularly as we pass from one lamina to the next. The surprising thing is that the chain directions should be preserved so well, not in adjacent, but in alternate layers, all the way through the cell wall. We have no satisfying explanation yet of this early, though striking enough, achievement in molecular architecture, but the mechanism can hardly involve orientation by deposition on cellulose chains already laid down, as was once thought. Some factor internal to the cellulose wall is indicated, some directional rhythm in the protoplasmic lining that synthesises the cellulose. *Valonia* is one of the lowliest of living creatures, and we have learnt much about its metabolism and wall structure—but we know nothing really.

This question of polysaccharide synthesis, aggregate formation and wall building is, of course, one of the great fundamental questions in molecular biology: the *Valonia* problem is only one aspect of it. Through it all runs the ubiquitous spiral, and underneath, dimly, we

can always glimpse the proteins, not alone, but always working in collaboration, with sugars, phosphoric acid, bases, nucleic acids. . . .

The result is an organisation, a structure with directional properties: and the value of the *Valonia* map lies not so much in itself, as in what it suggests. Its geometrical poles for instance—the points where the meridians and logarithmic spirals converge—appear to be peculiarly associated with the development of rhizoids, and we call to mind the polarity of *Fucus* eggs (86), the oriented chitin in the sporangiophores of *Phycomyces* (87), the polysaccharide or chitinous cysts of protozoa (88), the specific polysaccharide sheaths of the strains of pneumococcus, and a world of linings, membranes, coverings and the like, whose molecular architecture must fall into the same broad category as that of *Valonia*. Perhaps even Farr's concept (89) of cellulose being synthesised first as ellipsoidal particles—though, to be sure, they may be simply tactoids, like those formed by the tobacco mosaic virus—may be interpreted on a similar basis: the primary cellulose particle may be just another polysaccharide sheath round a protein nucleus.

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# THE CHEMISTRY OF THE ACYCLIC CONSTITUENTS OF NATURAL FATS AND OILS

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The reviewers regret that it is impossible in the available space to mention more than a small fraction of the material that has been published during the past year on the lipids of vegetable, animal or bacterial origin. Many important investigations dealing with synthetic compounds, fatty acids, alcohols, glycerides, phospholipids, etc., have been left out entirely. It is hoped that these omissions will receive adequate attention in subsequent volumes of this *Review*.

## VEGETABLE FATS

In the recent literature there are recorded a large number of analyses of various fats and oils of vegetable origin, but lack of space prevents detailed descriptions of all the important data that have been published. The original papers must be consulted by those interested since it is possible in this review to mention only what appear to be the most outstanding results.

Steger & van Loon (1) examined the oil obtained from the seeds of *Onguekoa gore* Engler. The air-dried kernels yielded 57.8 per cent of oil: saponification number 187, acetyl number 69, acid number 21.1, unsaponifiable matter 1.1 per cent and fatty acids 92.3 per cent with an iodine number of 232. The acids contained only 2.2 per cent of saturated acids. The unsaturated acids contained linolenic acid and a new unsaturated acid of the formula  $C_{18}H_{30}O_2$  which apparently contained 1 double bond and 1 triple bond. The same authors have studied the oil obtained from the seeds of *Valerianella olitoria* Poll., (2) which appears to be similar to the poppy, sunflower or soybean oil. The same authors (3) report an analysis of Po-Yoak oil prepared from the kernels from *Parinarium sherbroense*, a plant from Sierra Leone. The fat had the following constants:  $d_{78/4}$  0.9250, acid number 1.35, saponification number 192, Reichert-Meissl number 0.43, unsaponifiable matter 0.58 per cent, fatty acids 93.5 per cent. From an old sample of the fat the authors isolated, after saponification, 12 per cent of saturated acids,  $\beta$ -couepinic acid ( $C_{18}H_{28}O_8$ ), a keto-eleostearic acid,  $\beta$ -eleostearic acid and a small amount of oleic acid.

The oil extracted from raw and roasted coffee beans as well as that from used coffee grounds was analyzed by Bauer & Neu (4). The results would indicate that all three oils were very similar in composition.

Bömer & Hüttig (5) distilled in a high vacuum the fat isolated from the seeds of the babassu palm and purified the distillates by crystallization. The following glycerides were obtained in pure form: myristodilaurin, laurodimyristin and palmitodimyristin. The distillation residue contained glycerides of oleic, stearic, myristic, lauric and caprylic acids.

An extensive investigation of the composition of the oils obtained from the seeds of *Trifolium incarnatum* L., *Anthyllis vulneraria* L., *Ornithopus sativus* Link., and *Lotus corniculatus* L., was made by Fink & Richter (6). Similar studies have been reported on the oil from apple seed by Hokrova (7), on raspberry-seed oil by Marcelet (8) and on quince-seed oil by Pritzker & Jungkuntz (9). Rureš & Bednář (10) examined the oil from the kernel of *Fraxinus excelsior* L., and found 7 per cent of saturated acids, composed of palmitic and stearic acids. Unsaturated acids were represented by oleic, linoleic and linolenic acids.

Several new sources of fats and oils for Germany have been investigated. In this connection Josephs (11) examined freshly ground chufa tubers and found 14.4 per cent of oil. The latter contained 17.3 per cent of saturated acids, 67.6 per cent of oleic and 15.2 per cent of linoleic acids. With the same purpose in view Kaufmann & Fiedler studied the oil components of linden seed (12) and rocket-seed oil from *Eruca sativa* (13) while Kaufmann & Baltes (14) investigated the possibility of utilizing the oil from horse chestnuts. Horse chestnuts contain about 3.8 per cent of oil and the authors estimate that Germany might produce about 1000 tons of oil from this material annually. Kaufmann & Schmidt (15) investigated the composition of the fat from *Oidium lactis* (*Oospora lactis*) while Kaufmann, Baltes & Büter (16) have examined the fat from the seeds of *Trichosanthes cucumeroides*. In addition to trichosanic acid, an isomer of eleostearic acid, the fat contained mainly linoleic and oleic acids together with 8.6 per cent of saturated acids. Kaufmann & Baltes (17) report on the composition of Tsubaki oil obtained from the seed of *Camellia japonica* and found 10.6 per cent of saturated acids, 2.1 per cent of linoleic acid and 82.6 per cent of oleic acid. The oil is therefore recommended as a convenient source for prepar-

ing pure oleic acid. The same authors (18) have studied the properties of the fat from the fruit of the Butia palm (*Palma campestris*) and have found the following constants: acid number 0.5, saponification number 237.3, unsaponifiable 0.06 per cent, iodine number 48.7, thiocyanogen number 38.2, Reichert-Meissl number 4.3, Polenske number 8.8. As indicated by the last two values the fat contained a relatively large amount of lower volatile acids.

Comparative analyses of *Stillingia* oil of Chinese and American origin have been reported by Jamieson & McKinney (19). The chemical constants of the two oils were not strikingly different and the fatty acid components were similar. The saturated fatty acids, 6 to 8 per cent, consisted of palmitic, stearic and arachidic acids. The unsaturated acids, 88 to 85 per cent, consisted mainly of linoleic and linolenic acids together with a small percentage of oleic acid.

Comparative analyses have also been reported by Dollear, Krauczunas & Markley (20) on the composition of the oil of soy beans grown in different localities. The chemical composition of the oils from superior (Abyssinian) and inferior (Bison) flax seeds grown under different conditions have been reported by Gross & Bailey (21). Cook & Goodrich (22) have reported analyses of the oil from the pericarp and the seed of *Sambuccus calicarpa*. The composition and constants of the oil of papaya seed, *Carica papaya*, were determined by von Loesecke & Nolte (23). The fatty acids had the following percentage composition: palmitic 11.94, stearic 5.49, arachidic 0.32, oleic 79.94, and linoleic 2.22.

Cole & Cardoso (24) have investigated the fatty acids contained in chaulmoogra oil extracted from the seeds of *Carpotroche brasiliensis*, and found hydnocarpic 45.0, chaulmoogric 25.4, palmitic 6.6, gorlic 15.4, and oleic 6.3 per cent. The same authors (25) analyzed the oil from the seeds of *Oncoba echinata* and reported the following fatty acids: palmitic 7.8, oleic 2.2, gorlic 14.7, and chaulmoogric 74.9 per cent.

The oil of the hackberry, the fruit of *Celtis occidentalis* L., was analyzed by Schuette & Zehnpfennig (26). The kernels yielded 43.15 per cent of oil. The fatty acids found after saponification were stearic 4.9, oleic 16.5, linoleic 70.4 per cent, together with a trace of butyric acid. Schuette, Vogel & Wartinbee (27) have reinvestigated the composition of alfalfa-seed oil. The crushed seed yielded 8.63 per cent of oil on extraction with petroleum ether and the chemical constants of the oil were found to be similar to those reported by earlier investigators.

McKinney & Jamieson (28) have reported an analysis of the Ouricury palm-kernel oil. The oil had the following constants:  $d_{25/25}$  0.9221,  $n_D^{25}$  1.4543, saponification number 256.9, iodine number (Hanus) 14.69, thiocyanogen number 12.78, acid number 11.2, Reichert-Meissl number 5.93, Polenske number 18.38, unsaponifiable 0.27 per cent. The fatty acids, 78.9 per cent saturated and 14.2 per cent unsaturated, had the following percentage composition: caproic 1.66, caprylic 9.10, capric 7.64, lauric 42.70, myristic 8.43, palmitic 7.15, stearic 2.15, arachidic 0.096, oleic 12.18, and linoleic 2.04.

Mowrah fat, expressed from the seeds of *Madhuca* (*Bassia*) *latifolia*, used in the soap and candle industries in India and to some extent as an edible fat, has been analyzed by Hilditch & Ichaporia (29). The glycerides contained the following percentages of acids: palmitic 23.2, stearic 18.9, oleic 42.4, linoleic 13.4, and unsaponifiable 2.1 per cent. The component glycerides determined according to the usual procedure of Hilditch, and expressed in molar percentages, were as follows: dipalmitostearins 1, oleodipalmitins 1, oleopalmitostearins 27, palmitodioleins 41, and steardioleins 30.

A similar analysis of phulwara butter, the seed fat of *Madhuca butyracea*, was reported by Bushell & Hilditch (30). This fat is used in India to some extent for food and for soap production. The fat contained the following percentages of acids: palmitic acid 55.40, stearic acid 3.55, oleic acid 35.25, and linoleic acid 3.69. The glyceride components in molar percentages were oleodipalmitins 62, palmitodioleins 23, tripalmitin 8, and oleopalmitostearins 7. Shea butter was analyzed by the same procedure by Green & Hilditch (31). The fat had an acid number of 28 but the free acids were removed and only the neutral fat was examined. The following percentages of acids were found: palmitic 5.30, stearic 37.85, oleic 45.22, linoleic 3.95, and cinnamic 1.40. The component glycerides were represented in molar percentages as follows: steardioleins 45, oleodistearins 35, palmitodioleins 10, palmitostearins 5, trioleins 5.

A study of the composition of ground nut oil and sesame oil after progressive hydrogenation has been reported by Hilditch, Ichaporia & Jasperson (32). The following percentages of fatty acid components were obtained for the original sesame oil (results which agree with earlier reports): palmitic 8.98, stearic 4.25, arachidic 0.83, oleic 44.84, and linoleic 39.91; and for the ground nut oil: palmitic 8.31, stearic 3.05, arachidic 2.37, behenic 3.10, lignoceric 1.10, oleic

55.72, and linoleic 25.94. In both fats, when subjected to the usual analytical procedure, the estimated amount of tri- $C_{18}$  glycerides was quite close to the minimum limit, which would indicate that the component glycerides containing acids lower than  $C_{18}$  were combined with two molecules of the predominant  $C_{18}$  acids.

By employing the method of partial hydrogenation and subsequent analysis of the products formed, Hilditch & Thompson (33) were able to show in the case of olive oil and tea-seed oil that the tri- $C_{18}$  glycerides present in the original oils are close to the minimum theoretical values. The olive oil used in this investigation originated in Palestine and it contained the following percentages of fatty acids: myristic 0.49, palmitic 10.01, stearic 3.25, arachidic 0.09, oleic 77.44, linoleic 8.58. The possible limits for tri- $C_{18}$  glycerides in this oil would be from 69 to 84 molar per cent. The amount found was 70 molar per cent.

Bushell & Hilditch (34) have reported some interesting observations on the course of hydrogenation of various binary mixtures of mixed glycerides in order to determine the preferential rates of hydrogenation of such mixtures. In the hydrogenation of natural fats consisting largely of mixtures of palmito-di-unsaturated glycerides and tri-unsaturated glycerides, for instance oleolinoleins, etc., which occur in cotton-seed oil or in the depot fats of the pig, it has been found that the more saturated glycerides such as palmitodioleins pass largely into palmitostearins before tristearins are formed. The hydrogenation of known binary mixtures followed the same course as in the case of the natural oils, that is, the more saturated component of the mixtures was hydrogenated first.

The method of partial hydrogenation of various types of oils has been developed by Hilditch and his school with the object in view of elucidating the structure of triglycerides occurring in natural fats. A general review of the analytical operations was given by Harper, Hilditch & Terleski (35). It was found in general that partial hydrogenation resulted in complete saturation of monoethenoid acids, such as  $C_{14}$ ,  $C_{16}$ ,  $C_{18}$ , before the polyethenoid acids of the  $C_{18}$ ,  $C_{20}$ , or  $C_{22}$  series became saturated. The fats after hydrogenation to varying degrees of unsaturation were examined. The fully saturated glycerides were separated as neutral material after the unsaturated glycerides still present had been converted by the usual oxidation procedure to acidic compounds. The fatty acids contained in the fully saturated glycerides were separated into pure components and deter-

mined. After the procedure had been developed its application in the study of the composition of antarctic whale oil was described by Hilditch & Terleski (36) and of the component acids and glycerides contained in the North Sea cod-liver oil by Harper & Hilditch (37).

#### ANIMAL FATS

*Fats from land animals.*—Grossfeld (38) reports the following constants and composition of the tail fat of the fat-tailed sheep: m.p. 41.8°, saponification number 198.8, iodine number (Hanus) 53, thiocyanogen number 47.7, unsaponifiable 0.10 per cent, total fatty acids 95.4 per cent. The percentages of fatty acids were: oleic 47.1, linoleic 5.8, iso-oleic 2.5, total lower fatty acids 2.0 and butyric acid 0.4. Wool fat was examined by Heiduschka & Nier (39) who found 62.7 per cent of fatty acids and 32.8 per cent of unsaponifiable matter. The fatty acids were fractionally recrystallized, yielding cerotic and lanoceric acids, while ceryl alcohol was isolated from the unsaponifiable matter. The propyl, isopropyl, isobutyl and amyl esters of cerotic acid and the ethyl ester of lanoceric acid were prepared.

Dhingra & Sharma (40) have analyzed goat tallow according to the methods of Hilditch. The fat had the following constants: setting point 45.9°,  $n_{D}^{20}$  1.4564, saponification number 282, iodine number 33.5, acid number 2.1, unsaponifiable matter 0.18 per cent. The fatty acids, 58.1 per cent solid and 41.9 per cent liquid, had the following percentage composition: lauric 3.5, myristic 2.1, palmitic 25.5, stearic 28.1, arachidic 2.4, oleic 38.4. After the fat had been oxidized with permanganate in the usual manner the saturated glycerides, after purification by fractional crystallization, had the following molar percentage composition: tristearin 1 to 2, arachidostearins 3, tripalmitin 6 to 8, palmitodistearin 7 to 8, steardipalmitin 1 to 2.

Hilditch & Longenecker (41) report an extensive investigation of ox-depot fat, special attention being given to the identification of certain minor fatty acid constituents. The presence of small amounts of lauric and arachidic acids and also of tetradecenoic, hexadecenoic and arachidonic acids was shown. The constitution of the myristoleic acid was shown to be  $\Delta^{9:10}$ -tetradecenoic acid and of the palmitoleic acid to be  $\Delta^{9:10}$ -hexadecenoic acid. The acid components and glyceride structure of ox-depot fat were further investigated by Hilditch & Paul (42). They found that the fully saturated glycerides amounted to 17.4 per cent and contained in molar percentages tripalmitin 3.4, dipalmitostearin 7.8, palmitodistearin 5.8, and tristearin 0.4. The

unsaturated components were represented by oleo-disaturated glycerides, 49.0 per cent and dioleomonosaturated glycerides 33.6 per cent. Trioleins were not detected.

The composition of the fatty acids of milk fat from cows on a normal diet was determined by Hilditch & Longenecker (43). The acids volatile with steam amounted to 4.7 per cent, the solid acids to 48.5 per cent, and the liquid acids to 46.8 per cent. The following percentages of acids were found: butyric 3.03, caproic 1.39, caprylic 1.51, capric 2.68, lauric 3.71, myristic 12.01, palmitic 25.17, stearic 9.17, as arachidic 1.32,  $\Delta^{9:10}$ -decenoic 0.32,  $\Delta^{9:10}$ -dodecenoic 0.42,  $\Delta^{9:10}$ -tetradecenoic 1.58,  $\Delta^{9:10}$ -hexadecenoic 3.96, oleic 29.54, octadecadienoic 3.57, as arachidonic 0.28.

Longenecker & Hilditch (44) have reported comprehensive analyses of the fat from the carcasses of rats which had been fed on a whole milk diet.

A very comprehensive report was published by Hilditch & Shorland (45) on the component fatty acids present in the glycerides and phosphatides isolated from the liver fats of New Zealand farm animals. In general the phosphatides contained a larger proportion of stearic acid and of unsaturated  $C_{20}$  and  $C_{22}$  acids than the corresponding glycerides.

*Fats from marine animals.*—It has been shown in experiments reported by Tsujimoto & Koyanagi (46) that it is possible to separate the greater portion of the wax esters of the sperm whale head oil from the glycerides by distillation at about 2 mm. pressure and up to about 280°. This process of separation was extended to the pilot whale head oil (47) and to sperm blubber oil (48). It was found in each case that the glycerides remained largely in the distillation residue.

Ueno & Ishihara (49) have reported a study of ikanago fish oil. The ikanago, *Ammodytes personatus* Girard, is a small salt water fish and its oil appears occasionally on the market. The following constants were reported:  $d_{15/4}^4$  0.9365,  $n_{D}^{20}$  1.4849, saponification number 194.4, acid number 3.82, iodine number (Wijs) 169.1, unsaponifiable matter 0.83 per cent. The oil gave 22.8 per cent of solid and 77.2 per cent of liquid acids with an iodine number of 268.5. The liquid acids were found to contain large amounts of highly unsaturated  $C_{20}$  and  $C_{22}$  acids.

Tsujimoto (50) examined some of the constituent fatty acids in green turtle oil and found oleic acid as the chief component. In addition myristic, palmitic and stearic acids were present together with a



small amount of highly unsaturated  $C_{20}$  or  $C_{18}$  acids. The oil probably also contained lauric, dodecenoic, tetradecenoic and hexadecenoic acids.

The fat of the green turtle, *Chelone mydas* Linn., from Seychelles Islands, has been investigated by Green & Hilditch (51) who report the following constants: saponification number 265.9, acid number 3.2, iodine number 66.1, unsaponifiable matter 1.5 per cent. The fat contained about 10 per cent of fully saturated glycerides consisting chiefly of lauro- and myristo-palmitins and some tri- $C_{18}$  glycerides. The percentages of fatty acids were: lower acids 0.2, lauric 13.3, myristic 10.6, palmitic 17.0, stearic 4.1, as tetradecenoic 1.3, hexadecenoic 7.8, oleic 39.6, higher unsaturated acids (chiefly  $C_{20}$  and  $C_{22}$ ) 6.1.

The fatty acids of liver oils and of phosphatides of some New Zealand fish have been investigated by Shorland & Hilditch (52). In the case of the proper fish taken during spring, early and late winter the liver oil showed a marked variation in vitamin-A content, iodine number and in unsaponifiable matter and the phosphatide content varied from a trace in spring to 18.8 per cent in winter.

Lovern (53) has investigated the effect of food on the composition of the depot fat of eels. A diet consisting of mussels, containing about 1.1 per cent of fat, had no detectable effect on the depot fat but on a diet of herring, containing about 20.7 per cent of fat, the ingested fat was incorporated to some extent in the depot fat with but little change in the relative proportions of the various fatty acids. The same author has also investigated seasonal changes in the composition of herring fat (54).

#### PHOSPHOLIPIDS

Investigations dealing with the purification and composition of phospholipids continue to interest biological chemists. Many publications on the phospholipids have appeared during the past year but only a few can be mentioned in this review.

A comparative study of the phospholipids of barley, wheat and oats was reported by Diemair, Bleyer & Schmidt (55). The yield was from 0.12 to 0.16 per cent of the grain. The analytical values for nitrogen, about 1.0 per cent, and for phosphorus, about 2.2 per cent, were low but the products were stated to be free from carbohydrate. The lecithins were precipitated as cadmium chloride—double salts and separated into  $\alpha$ - and  $\beta$ -lecithins.

An improved method for the separation and purification of  $\alpha$ - and  $\beta$ -lecithins and cephalins was described by Yoshinaga (56). The same author (57), by the use of his improved method of separation, determined the proportions of  $\alpha$ - and  $\beta$ -lecithins and  $\alpha$ - and  $\beta$ -cephalins in various tissues of rabbits and dogs.

The preparation and analysis of phospholipids from rape seed was reported by Rewald (58) and also by Heiduschka & Neumann (59). Belozerskii & Kornev (60) studied the composition of the phospholipids in the embryos and cotyledons of soy beans, and Shabanov (61) has reported on the phospholipids of tobacco seeds.

A detailed study of the component fatty acids present in the phospholipids of soy beans and rape seeds has been reported by Hilditch & Pedelty (62). The chief saturated acid was palmitic acid and the principal unsaturated acid was linoleic acid but small amounts of hexadecenoic, oleic and linolenic acids were found. The rape seed phospholipids also contained erucic acid.

An analysis of the fatty acids present in the lecithin and glycerides of egg yolk was carried out by Riemenschneider, Ellis & Titus (63). The distribution of fatty acids in the lecithin fraction was reported as follows: palmitic 31.8, stearic 4.1, oleic 42.6, linoleic 8.2, and clupanodonic acids 13.3 per cent. The percentages of glyceride fatty acids were as follows: lower than palmitic 0.7, palmitic 25.2, stearic 7.5, palmitoleic 3.3, oleic 52.4, linoleic 8.6, and clupanodonic 2.3.

The composition of sphingomyelin isolated from normal beef spleen has been reinvestigated by Thannhauser & Benotti (64) while the composition of sphingomyelin from the stromata of red blood cells has been reported by Thannhauser, Setz & Benotti (65). The composition of the cerebrine of beer yeast was investigated by Ruppel (66) who reported that the substance on hydrolysis yields only sphingosine and a hydroxy acid,  $C_{28}H_{56}O_8$ .

A method for the estimation of cephalin in small amounts of phospholipids was proposed by Williams, Erickson, Avrin, Bernstein & Macy (67): total phosphorus is determined and choline is determined by the micro method of Beattie (68); from the choline phosphorus ratio the cephalin content can be calculated.

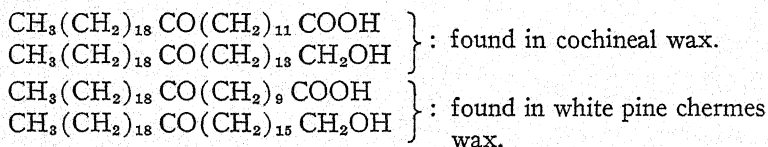
In connection with the problem of possible compound formation between phospholipids and proteins some interesting observations have been reported by Chargaff (69). He found that cephalin formed an apparently stable compound with salmine and that lecithin and sphingomyelin did not form such compounds. The same author (70)

has described the isolation from the spleen, in a case of Niemann-Pick's disease, of a lipid fraction which inhibited blood clotting. The substance contained 7.5 per cent of nitrogen, 3.3 per cent of phosphorus and 2.7 per cent of sulfur.

#### THE COMPONENTS OF WAXES

Our knowledge of certain wax components such as alcohols, ketones, and acids, has been greatly enlarged through the important and fundamental investigations of Chibnall and his collaborators. x-ray analyses of the purified cleavage products have been found of great value in determining the chain length as well as the position of active groups and in elucidation of chemical structure.

Blount, Chibnall & el Mangouri (71) have examined the wax of white pine chermes, *Adelges (Pineus) strobi* Börner. Earlier investigations had indicated that the components liberated on saponification were similar to but not identical with those obtained from cochineal wax. The cleavage products have now been identified as 17-keto-*n*-hexatriacontanol and 11-keto-*n*-triacontanoic acid together with a negligible amount of what may be called normal fatty acids. In order to show the relation between the waxes of cochineal and white pine chermes the formulas of their respective alcohols and acids are given below:



The new keto acid melted at 103.3–103.6°, its oxime at 62.5°. The new keto alcohol melted at 102–102.5°, its oxime at 78.5° and its acetyl derivative at 84.2°. The keto acid derived from the keto alcohol by oxidation with chromic acid melted at 107.5–108°.

The chain length of the two new products was foretold at once by x-ray analysis and their constitutions were determined, as in the case of the products from the cochineal wax, by putting the oximes of the respective ketonic acids through a Beckmann transformation and hydrolyzing the resulting mixed amides.

The wax from the leaves of sandal, *Santalum album* Linn., has been examined by Chibnall *et al.* (72). The wax was extracted from sandal leaves with hot 95 per cent alcohol and was purified by precipitation from alcohol and acetone. It was a pale yellow solid, m.p. 86°. On saponification the wax yielded 16.3 per cent of higher

fatty acids, m.p. 80.5°, and 74.3 per cent of unsaponifiable matter. The latter was separated by the usual procedure into primary alcohols, about 50 per cent, palmitone 44 per cent, and 6 per cent of *d*-10-hydroxypalmitone. The primary alcohols, m.p. 82.5–83°, (acetate, m.p. 65.7°; derived acid, m.p. 86–86.2°) represented a mixture consisting of about 75 per cent *n*-octacosanol and 25 per cent *n*-triacontanol.

Palmitone [ $\text{CH}_3(\text{CH}_2)_{14}\text{CO}(\text{CH}_2)_{14}\text{CH}_3$ , m.p. 82.8–83°, oxime m.p. 59°] and *d*-10-hydroxypalmitone [ $\text{CH}_3(\text{CH}_2)_{14}\text{CO}(\text{CH}_2)_5\text{CHOH}(\text{CH}_2)_8\text{CH}_3$ , hexagonal plates, m.p. 96.4–96.6°; acetate, m.p. 43–43.5°; oxime, m.p. 63.4–63.6°] are of particular interest because they have not previously been found in waxes.

A method for the separation of carbonyl compounds from the unsaponifiable fraction of waxes containing ketones, primary alcohols and paraffins has been described by el Mangouri (73): the mixed unsaponifiable fraction is refluxed in 95 per cent alcohol with  $\beta$ -carboxyphenylhydrazine and a few drops of pyridine, after which the hydrazone is precipitated as the barium salt and the ketone is regenerated by refluxing with an alcoholic solution of formaldehyde. *n*-Nonacosan-15-one was separated from cabbage leaf wax, and palmitone and *d*-10-hydroxypalmitone were separated from sandal leaf wax by this procedure.

The composition of the wax obtained from cork was reported by Zetzsche & Luscher (74) and Wagner (75) reports an examination of the wax obtained from coffee beans in the Kaffee-Hag process. The presence of eicosanol and eicosenol in sperm blubber oil was reported by Toyama (76). Two new alcohols, lano-octadecyl alcohol ( $\text{C}_{18}\text{H}_{38}\text{O}$ , m.p. 42–43°) and lanyl alcohol [ $\text{C}_{21}\text{H}_{40}(\text{OH})_2$ , m.p. 78°] have been described as constituents of wool wax by Kuwata & Katuno (77). A study of the wax produced by the Japanese coccid, *Sasakiaspis pentagona* Tar., by Kono & Maruyama (78) would indicate that the chief constituents are melissyl alcohol, melissic acid and cerotic acid.

An investigation of the petroleum-ether and ether-soluble constituents of grape pomace by Markley, Sando & Kendricks (79) showed that the petroleum-ether-soluble constituents consisted of glycerides, waxes and hydrocarbons. The unsaponifiable material contained sitosterol, the hydrocarbons, nonacosane and hentriacontane, and higher primary alcohols. The ether-soluble constituents, obtained subsequent to extraction with petroleum ether, consisted

principally of oleanolic acid,  $C_{30}H_{48}O_3$ . Certain unidentified resinous substances were also present.

#### METHODS

Modifications and improvements in methods useful in fat analyses have been suggested in several publications. A few of these will be mentioned.

The application of molecular distillation to the separation of the esters of highly unsaturated acids of fish oils has been discussed by Farmer & van den Heuvel (80). These authors point out that molecular distillations carried out in high vacuum and at relatively low temperatures reduce the chances for rearrangements in the double bond systems.

Hafner, Swinney & West (81) have recommended an improved method for the determination of hydroxylated acids of fats. A micro-method for the determination of the saponification number of fats has been published by Furter (82). Kaufmann & Hartweg (83) have reported a semi-micromethod for the determination of the thio-cyanogen number. Kirk has described improvements in the methods for the determination of cerebrosides (84) and of phospholipids (85) in various tissues.

Shinowara & Brown (86) have reported the successful alcoholysis of beef suprarenal phospholipids and the preparation of methyl, ethyl, *n*-propyl, *n*-butyl, and *n*-amyl esters of the fatty acids by refluxing with the respective alcohols containing either 5 per cent hydrochloric acid or 7.5 to 12 per cent sulfuric acid. The octabromides of the arachidonic fractions of the esters were separated by bromination.

The limitations inherent in the usual methods for the separation of saturated and unsaturated fatty acids of fats and oils have been discussed, and improved procedures have been suggested by Stillman & Andrews (87) and by Pelikan & von Mikusch (88). The estimation of oleic, linoleic and linolenic acids in the presence of one another has been discussed by Delvaux (89). An improved technique for the Kreis test has been developed by Walters, Muers & Anderson (90).

A study of the heating and cooling curves of  $\alpha$ - $\alpha'$ -diglycerides, reported by Malkin, el Shurbogy & Meara (91), would indicate that the  $C_{12}$ - $C_{15}$  diglycerides occur in 3 forms as do the monoglycerides, whereas the  $C_{16}$ - $C_{18}$  diglycerides occur in only two forms, resembling in this respect the triglycerides. The results of x-ray analyses of  $\alpha$ - $\alpha'$ -diglycerides favor the view that crystals are built up of layers of double molecules with the two hydrocarbon chains lying parallel on the same side of the glycerol molecule.

## LIPIDS OF BACTERIAL ORIGIN

Recent studies on the so-called wax fractions of acid-fast bacteria have shown that these substances are of very complex composition representing mixtures of solid glycerides, esters of higher alcohols with higher acids, and esters of higher acids with carbohydrates and specific polysaccharides.

The wax isolated from the avian tubercle bacillus was studied by Reeves & Anderson (92). The purified wax was a white powder, m.p. 54–55°, iodine number 4.5,  $[\alpha]_D$  in chloroform +38.6°. Composition: C 75.38, H 12.14, O (by difference) 12.48 per cent. Saponification yielded about 84 per cent of fatty acids, 10 per cent of unsaponifiable matter, and 13 per cent of carbohydrate. The fatty acids represented mainly a mixture of optically active hydroxy acids of high molecular weight corresponding approximately to the formulas  $C_{38}H_{74}O_3$  and  $C_{88}H_{174}O_3$ . A small amount of lower acids was also present but could not be identified. None of the ordinary saturated or unsaturated fatty acids could be found. The hydroxy acids referred to above were strongly acid-fast.

The unsaponifiable matter contained no sterols but consisted of two higher secondary alcohols,

*d*-eicosanol-2 [ $CH_3(CH_2)_{17}CHOHCH_3$ ; m.p. 62–63°;

$[\alpha]_D$  in ether, +6.7°] and *d*-octadecanol-2 [ $CH_3(CH_2)_{15}CHOHCH_3$ ; m.p. 53–54°;  $[\alpha]_D$  in chloroform, +4.8°].

The carbohydrate on purification by means of basic lead acetate and acetylation yielded an octa-acetate (colorless crystals; m.p. 97–98°;  $[\alpha]_D$  in chloroform, –163.7°). Saponification of the acetyl derivative gave the pure carbohydrate as large colorless crystals (m.p. 98°;  $[\alpha]_D$  in water, +178°). The properties identify the carbohydrate as the disaccharide trehalose,  $C_{12}H_{22}O_{11} \cdot 2H_2O$ . Glycerol could not be detected.

*Mycolic acid*.—The principal ether-soluble constituent of the wax from the human tubercle bacillus was shown by Anderson (93) some years ago to be a hydroxy acid of high molecular weight which was designated by the term “unsaponifiable wax.” The “unsaponifiable wax” as shown by Stodola, Lesuk & Anderson (94) yielded on prolonged saponification a small amount of lower fatty acids, neutral material, and a hydroxy methoxy acid to which the name mycolic acid was given.

The neutral or unsaponifiable material contained no sterols but con-

sisted of the dihydroxy monomethoxy alcohol, phthiocerol ( $C_{35}H_{72}O_3$ ), which has been described as a constituent of the human tubercle bacillus wax by Stodola & Anderson (95).

Mycolic acid is obtained as a snow white powder consisting of fine globular particles (m.p.  $54-56^\circ$ ;  $[\alpha]_D$  in chloroform,  $+1.8^\circ$ ) and its composition corresponds approximately to the formula  $C_{88}H_{172}O_4$  or  $C_{88}H_{176}O_4$ . Mycolic acid is acid-fast. When mycolic acid is heated under reduced pressure to  $250$  to  $300^\circ$  it decomposes yielding a crystalline distillate which consists of *n*-hexacosanoic acid,  $C_{26}H_{52}O_2$ , and a non-volatile, nearly colorless, unsaturated residue.

Wiegand & Anderson (96) purified and analyzed wax fractions isolated from the mother liquors remaining from the purification of the phosphatide from the human tubercle bacillus. The purified wax was a white amorphous powder (m.p.  $45-46^\circ$ ;  $[\alpha]_D$  in chloroform,  $-0.8^\circ$ ) and it contained 0.3 per cent of phosphorus. On mild saponification the wax yielded glycerol, carbohydrate, unsaponifiable matter, fatty acids and a product corresponding to the so-called "unsaponifiable wax." The carbohydrate consisted of inositol and mannose. The fatty acids consisted of palmitic, stearic and hexacosanoic acids, unsaturated fatty acids of the  $C_{26}$  series, and liquid saturated fatty acids such as tuberculostearic acid,  $C_{19}H_{38}O_2$ , and phthioic acid ( $C_{26}H_{52}O_2$ ; m.p.  $20^\circ$ ;  $[\alpha]_D$  in ether,  $+11^\circ$ ). A levorotatory acid ( $C_{31}H_{62}O_2$ ;  $[\alpha]_D$  in ether, from  $-7.2^\circ$  to  $-10.4^\circ$ ) was also present. The unsaponifiable matter contained no sterols but consisted mainly of the alcohol phthiocerol,  $C_{35}H_{72}O_3$ . The "unsaponifiable wax" on prolonged saponification gave: mycolic acid; tuberculostearic acid,  $C_{19}H_{38}O_2$ ; the levorotatory acid ( $C_{31}H_{62}O_2$ ;  $[\alpha]_D$  in ether,  $-9.5^\circ$ ); and the alcohol phthiocerol,  $C_{35}H_{72}O_3$ . The mycolic acid corresponded in properties and composition to the mycolic acid described by Stodola, Lesuk & Anderson (94) and on pyrolysis it gave *n*-hexacosanoic acid and a neutral non-volatile residue.

The wax isolated from the bovine tubercle bacillus has been purified and analyzed by Cason & Anderson (97). The purified wax was a white amorphous powder (m.p.  $47-54^\circ$ ;  $[\alpha]_D$  in benzene,  $+15.6^\circ$ ; iodine number 3.2). It contained phosphorus 0.3 per cent and nitrogen 0.16 per cent. On mild saponification of the purified wax the following percentages of cleavage products were obtained: carbohydrate 9.02, glycerol 1.33, fatty acids 19.40, unsaponifiable matter 5.38, and a product corresponding to "unsaponifiable wax" 61.03. The carbohydrate contained phosphorus and consisted of a mixture of organic



phosphoric acids, including glycerophosphoric acid and a neutral phosphorus-containing polysaccharide. The latter, on hydrolysis with dilute sulfuric acid, gave mannose, inosite and inosite monophosphoric acid. The unsaponifiable matter contained no sterols but consisted mainly of a dihydroxy monomethoxy alcohol identical with phthiocerol (98) which had been isolated from the human tubercle bacillus wax by Stodola & Anderson (95). The "unsaponifiable wax" on prolonged saponification gave a mixture of high molecular weight optically active hydroxy acids, certain lower fatty acids, and phthiocerol. The optically active hydroxy acids consisted of a mixture from which no definitely pure acids could be isolated. The main fraction corresponded in properties to mycolic acid (94) and was named bovine mycolic acid (m.p. 56–58°;  $[\alpha]_D$  in chloroform, +2.7°; mol. wt. by titration 1219). On pyrolysis bovine mycolic acid gave *n*-hexacosanoic acid and a neutral non-volatile residue. The lower fatty acids on separation by the lead salt-ether method gave solid saturated acids and liquid acids with an iodine number of 5.

The solid saturated acids were separated by fractional distillation of the methyl esters into palmitic acid ( $C_{16}H_{32}O_2$ ; m.p. 62–63°) and a tetracosanoic acid ( $C_{24}H_{48}O_2$ ; m.p. 76–77°). The crystal form, diamond-shaped crystals or boat-shaped needles, differ from that of ordinary fatty acids. This fact and the low melting point would suggest that the acid is a branched chain acid.

The liquid acids, iodine number 5, were hydrogenated and again separated by the lead salt-ether method. The small amount of solid reduced acid was a mixture from which no pure substance could be isolated. The ether-soluble lead salts yielded liquid saturated acids which on fractional distillation of the methyl esters were separated into Fraction I and Fraction II.

Fraction I proved to be a new optically inactive branched chain acid isomeric with stearic acid. The physical properties of the free acid and of some of its derivatives are: free acid,  $C_{18}H_{36}O_2$ , crystalline solid, m.p. 29–30°; methyl ester,  $C_{19}H_{38}O_2$ , b.p. 112–114° at 0.006 mm., m.p. 0°,  $n_D^{25}$  1.4436; tribromoanilide, snow white needles, m.p. 96–96.5°.

Fraction II was no doubt a mixture. The ester on saponification gave an acid, a wax-like solid, m.p. 33–34°,  $[\alpha]_D$  –3.9°, eq. wt. 430, from which no pure tribromoanilide could be obtained.

It is noteworthy that the only ordinary fatty acid that could be isolated from the bovine tubercle bacillus wax was palmitic acid.



In general the chemical composition of the bovine tubercle bacillus wax resembles the wax obtained in the purification of the phosphatide from the human tubercle bacillus (96). The carbohydrate is similar in composition. The bovine mycolic acid closely resembles the mycolic acid present in the wax of the human tubercle bacillus. The waxes from both organisms contain the same higher alcohol, phthiocerol, but this alcohol has not been found in any of the other acid-fast bacteria. The results of the chemical analyses indicate therefore a similarity between the human and bovine tubercle bacilli that is in agreement with the pathogenicity of these organisms.

*The firmly bound lipids of the tubercle bacillus.*—While it has been known for a long time that the tubercle bacillus and other acid-fast bacteria contain considerable percentages of lipids that could not be extracted by neutral solvents, yet nothing was known concerning the chemical composition of this material until very recently. In a study reported by Anderson, Reeves & Stodola (99) it was shown that the human tubercle bacillus which had been freed completely of all lipids soluble in neutral solvents yielded from 12 to 15 per cent of additional ether-soluble lipids after the partly defatted cells had been treated with an acidified alcohol-ether solution. A portion of this lipid material exhibited the peculiar property of not passing through a Chamberland filter. The filtrable as well as the unfiltrable lipid was easily soluble in ether, chloroform and benzene but practically insoluble in alcohol or acetone and both fractions were apparently closely related to the tubercle bacillus wax.

The unfiltrable lipid was obtained by precipitation with alcohol from ethereal solution as a white amorphous powder, m.p. with decomposition about 200°. The substance contained C 64.2, H 10.2, P 0.20 and N 0.40 per cent. Saponification gave about equal parts of mycolic acid and a polysaccharide together with a very small amount of fatty acids.

The polysaccharide gave a precipitin reaction with immune serum in dilutions up to 1:1,000,000 and it also gave pentose color reactions. After hydrolysis the maximum reduction amounted to 57 per cent calculated as glucose. The reducing sugars consisted of *d*-mannose 6.6, *d*-arabinose 38.7 and *d*-galactose 12.2 per cent. The rest of the molecule could not be identified.

The filtrable portion of the firmly bound lipids contained about 25 per cent of a polysaccharide similar to the polysaccharide described above, 41 per cent of mycolic acid and 35 per cent of lower acids. The

lower acids consisted of palmitic and stearic acids, unsaturated  $C_{16}$  and  $C_{18}$  acids together with the branched chain saturated tuberculostearic acid.

Anderson, Lothrop & Creighton (103) examined the carbohydrate, manninositose, which is contained in the phosphatide of the human tubercle bacillus. When a benzene solution of the phosphatide at room temperature was mixed with a dilute solution of alcoholic potassium hydroxide the fatty acids were split off completely and a mixture consisting of organic phosphoric acids and a phosphorus-containing carbohydrate separated from the solution. The carbohydrate was dephosphorylated by heating with dilute ammonium hydroxide in a sealed tube to  $170^{\circ}$  for 8.5 hours. After removing the phosphoric acid, the carbohydrate, which did not reduce Fehling's solution, was isolated and purified by acetylation. The purified carbohydrate was obtained as a white amorphous powder on saponifying the acetyl derivative, but it could not be obtained in crystalline form. The carbohydrate, named manninositose, gave on hydrolysis 2 parts of mannose and 1 part of inosite. Manninositose is evidently a glycoside composed of 2 molecules of mannose combined with 1 molecule of inosite.

Kasuya (105) has described the isolation and identification of  $\beta$ -hydroxy-*n*-butyl aldehyde from the acetone-soluble fat of the human tubercle bacillus and also from the culture medium on which the bacilli were grown. The author reports in the same paper that the aldehyde gives a typical tuberculin reaction when injected under the skin.

Wagner-Jauregg (106) has described the isolation of fatty acids from the bacterial residues from the tuberculin manufacture. The types of bacilli are not given. A liquid saturated acid identical with tuberculostearic acid was obtained and also certain other optically inactive liquid saturated fatty acids from which a crystalline tribromoanilide was prepared (m.p.  $66-68^{\circ}$ ) which corresponded to the formula  $C_{25}H_{50}NOBr_3$ . Umezu & Wagner-Jauregg (107) have prepared phosphatide, fat and wax fractions from the human strain of tubercle bacilli. A wax-like material of low melting point,  $35-40^{\circ}$ , which was free from phosphorus was separated in the purification of the phosphatide. This fraction on saponification gave a neutral substance, apparently identical with phthiocerol, and a substance corresponding to the so called "unsaponifiable wax," m.p.  $57-58^{\circ}$ .

Kraut & Burger (108) have carried out experiments to determine whether lipases from various sources, human serum, horse serum, blood, etc., were capable of hydrolyzing the acetone-soluble fat of the

human tubercle bacillus. The results would indicate that the leucocytes contain the most active lipase.

An analysis of the fat soluble in ice cold acetone, isolated from *Bacillus leprae*, has been reported by Anderson, Reeves & Crowder (100). The fat contained 26.2 per cent of free fatty acids which were removed and examined separately. The neutral fat was a dark, red-colored, semisolid mass with the following constants: m.p. 19–20°, iodine number 47.9, Reichert-Meissl number 5, Polenske number 2, unsaponifiable matter 22.1 per cent. On saponification it gave 75.7 per cent of fatty acids.

The fat was not a glyceride. After saponification the only water-soluble product that could be isolated was the disaccharide trehalose,  $C_{12}H_{22}O_{11} \cdot 2H_2O$ . The fat is therefore an ester of fatty acids with trehalose, being similar in this respect to the fat of the human tubercle bacillus (101).

The unsaponifiable matter contained no sterols but two new alcohols,  $\alpha$ -leprosol, m.p. 100–101°, and  $\beta$ -leprosol, m.p. 84–85°, which were obtained as described by Crowder, Stodola & Anderson (102). The leprosols are identical in composition and correspond to the formula  $C_{26}H_{46}O_2$  or  $C_{25}H_{44}O_2$  but they differ in solubility and melting point. Both substances are monohydroxy, monomethoxy alcohols and possess phenolic properties. They form slightly soluble addition compounds with digitonin but they give no sterol color reactions. The free leprosols are easily brominated forming crystalline monobromoderivatives but the acetyl derivatives are completely indifferent to bromine.

The fatty acids were a complex mixture of solid saturated, solid unsaturated, liquid unsaturated and saturated branched chain optically active acids. The ordinary saturated fatty acids were represented by caproic, myristic, palmitic, stearic, arachidic, behenic and tetracosanoic acids. The unsaturated acids belonged to the  $C_{14}$ ,  $C_{16}$ ,  $C_{18}$ ,  $C_{20}$ ,  $C_{21}$ ,  $C_{22}$  and  $C_{25}$  series. Several new dextrorotatory branched chain acids were also found, namely  $C_{16}H_{32}O_2$ ,  $C_{19}H_{38}O_2$ , and  $C_{22}H_{44}O_2$ .

Chargaff & Levine (104) have reported examination of the lipids extracted from the crown gall bacterium, *Phytoplasma tumefaciens*. The organism, grown on a bean-broth medium, contained 5.3 to 5.8 per cent of acetone-soluble fat, 1.6 to 3.2 per cent of phosphatide, and 0.8 per cent of chloroform-soluble wax. The phosphatide after purification contained 3.2 per cent of phosphorus and 2.3 per cent of nitrogen. The acetone-soluble fat was saponified and the cleavage

products were examined. The fatty acids consisted of a mixture containing palmitic acid, oleic acid, unsaturated acids of high molecular weight and liquid saturated acids corresponding in molecular weight to the formula  $C_{21}H_{42}O_2$ . The unsaponifiable matter gave sterol color reactions but the sterol fraction might have been derived from the medium. The water-soluble component contained glycerol but no carbohydrates.

#### LIPIDS FROM FUNGI

Bernhauer & Posselt (109) have examined the fat extracted from *Aspergillus niger*. The fat, amounting to 0.34 per cent of the dried mycelium, had the following constants: acid number 71.2, saponification number 169, ester number 97.8, iodine number 95.1, Reichert-Meissl number 0.99, and Polenske number 0.7. On saponification the fat gave 67.5 per cent of fatty acids, 12.0 per cent of unsaponifiable matter, and 6.2 per cent of glycerol. The saturated fatty acids amounted to 13.0 per cent and consisted of palmitic, stearic and lignoceric acids. The unsaturated acids, 45.4 per cent, were represented by nearly equal parts of oleic and linoleic acids. The unsaponifiable matter contained a small percentage of ergosterol and a large amount of unidentified constituents.

Peck & Hauser (110) have reported a study of the lipids of the pathogenic fungus, *Blastomyces dermatiditis*, with the object of discovering any peculiar components that might be responsible for the pathogenic properties of the organism.

The organism, grown on a synthetic medium, yielded about 9.0 per cent of lipids soluble in alcohol-ether and some 0.6 per cent soluble in chloroform. The alcohol-ether soluble lipids were fractionated into phosphatide and acetone-soluble fat. The phosphatide contained 3.89 per cent of phosphorus and 1.78 per cent of nitrogen and after hydrolysis yielded 65 per cent of fatty acids, 3.0 per cent of unsaponifiable matter and 32.0 per cent of water-soluble material. The fatty acids were separated into 12.4 per cent of solid acids and 52.6 per cent of liquid acids. The solid acids consisted of palmitic and stearic acids while the liquid acids were composed of oleic and linoleic acids. Liquid saturated fatty acids similar to those occurring in the phosphatides of acid-fast bacteria were absent. The water-soluble constituents consisted of glycerophosphoric acid, choline and aminoethyl alcohol. The cleavage products were therefore identical with those found in the usual mixtures of lecithin and cephalin. A slight amount of carbohydrate was present but its nature could not be determined.

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# THE CHEMISTRY OF PROTEINS AND AMINO ACIDS

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Interest in the field of protein chemistry is growing rapidly and extends to many branches of biochemistry, as the fundamental rôle of proteins in many biological processes is more and more realised, especially in connection with the identification as proteins of many substances of specific activity. Recent discoveries and theories regarding the structure of the protein molecule have also stimulated research in this field. Reference should be made to a number of important conferences on proteins which were held during the year: at Cold Spring Harbor (published as *Cold Spring Harbor Symposia on Quantitative Biology* 6, 1938);<sup>1</sup> at Stuttgart in connection with the twelfth annual meeting of the Kolloidgeellschaft [published in *Kolloid-Z.* 85, 113-342 (1938)]; and at the Royal Society in London (a summary has been published by McFarlane). A monograph on amino acid and protein chemistry with many valuable contributions by different authors has been edited by C. L. A. Schmidt. A new edition of Jordan-Lloyd's book on protein chemistry has also appeared.

In accordance with a wish expressed by the Editors of this volume, this year's review will be devoted mainly to certain physicochemical properties of the proteins. As two of the most important methods of studying protein structure, namely x-ray analysis and enzymatic hydrolysis are dealt with in separate contributions to this volume (cf. Astbury, p. 113 and Linderstrøm-Lang, p. 37) the corresponding parts of this review have been abridged considerably.

## SIZE, SHAPE AND STABILITY OF PROTEIN MOLECULES

Svedberg and collaborators have continued their molecular weight determinations, and Svedberg (1 to 4) has published summaries of this work with tables of molecular constants as determined in the Upsala laboratory. These tables illustrate strikingly a fact which is to be considered as one of the most important results of this work, namely the rule of simple multiples for the molecular weights of proteins of widely different origin; this rule now seems to be firmly established, and is playing an important rôle in the recent theories of protein structure advanced by Bergmann and by Wrinch (cf.

<sup>1</sup> At the time of concluding this review only part of the papers presented at this Symposium were available to the author.



p. 171 and p. 176). It should be observed that at least four well-defined proteins have been found with a molecular weight of about 17,600, which must now be recognized as the ultimate unit instead of 35,000 to 36,000. Whatever the explanation for the rule of multiples may be, it seems more likely to be a question of the number of amino acid residues or of the "backbone" structure of the peptide chains than of an exact rule for molecular weights. Hence, in accordance with the differences of weight for the amino acid side chains a margin for deviations from the rule should be accepted.

Another important discovery, which was the result of the first ultracentrifugal observations, and has been confirmed since on a very large number of well-defined proteins, was their homogeneity in molecular size. In striking contrast to artificially prepared colloids, *e.g.*, gold sols, native soluble proteins are either monodisperse or paucidisperse, that is they contain either a single or a few molecular species, well defined with regard to mass and shape. These observations have clearly demonstrated the sharply defined individuality of the protein molecule. On the other hand, proteins occurring in the organism as more or less insoluble deposits, such as keratin, fibroin, and myosin, do not form definite molecules in solution. This, of course, does not disprove the existence of definite molecular entities in certain protein fibres, as assumed by Astbury (1), but may only be a consequence of our inability to find suitable solvents which would allow the characteristic molecular size to manifest itself unchanged in solution. Determination of lattice-cell dimensions of protein crystals by x-ray analysis offers an independent method of determining a maximum molecular weight, as the cell must contain at least one molecule. In a number of papers (Crowfoot & Riley; Crowfoot & Fankuchen; Bernal, Fankuchen & Perutz; Bernal, Fankuchen & Riley) the molecular weights determined by the ultracentrifuge method have been confirmed, as a whole, and the unit cell has been shown to contain one or a low number of molecules [cf. also *Ann. Rev. Biochem.*, 4, 108 (1935)]. The swelling and shrinking of the crystals and the uncertainty regarding the correct value for the density, which is necessary for such a comparison, seem to offer some difficulties (Bernal, Fankuchen & Riley).

The ultracentrifuge is capable of yielding valuable information regarding the size homogeneity of high molecular substances on account of the partial separation in the centrifuge cell of the differently sedimenting molecular species. This method of "sedimentation

analysis" has led to the discovery of the pH-stability regions of the proteins, and of interesting dissociation and association phenomena; it has also been of great help in the characterisation of protein mixtures by identification and quantitative determination of their components, as well as in serving as a guide in the search for suitable preparation methods. A number of recent papers have given important results. Eriksson-Quensel has reinvestigated in detail the interesting and well-defined proteins, phycoerythrin and phycocyan; the latter which has a molecular weight of 273,000 dissociates into halves at neutral reaction. The hemocyanins, which show very striking dissociation phenomena at the limits of their pH-stability regions, where they give rise to submultiples, continue to form convenient objects for studies of this kind; the high sedimentation velocity of these giant molecules and of their split products makes them particularly suited for ultracentrifugal analysis. Brohult has found that similar dissociations may be produced by ultrasonic waves, and Svedberg & Brohult found ultraviolet light capable of splitting the *Helix* hemocyanin molecule into halves. The influence of ultrasonic waves on proteins has also been studied by Thieme and by Freundlich & Gillings, and the effect of sound waves of audible frequencies by Chambers & Flosdorf. Of these dissociations, at least that which is produced by change of pH seems to be reversible to a large extent. Evidently these huge molecules are aggregates of smaller particles, held together by comparatively weak forces. The marked effect of some divalent cations on the dissociation of hemocyanins as studied by Brosteaux and by Eriksson-Quensel [quoted by Svedberg (4)] is possibly a clue to the nature of these forces [see also Wrinch (3)]. It seems probable that the interaction is highly specific for each protein, nevertheless Tiselius & Horsfall were able to demonstrate that the dissociation and reassociation by pH changes in a mixture of two different but biologically related hemocyanins (from *Helix pomatia* and *Helix nemoralis*) may produce new "mixed" hemocyanin molecules by cross combination of the dissociation products.

Dissociation reactions produced by other more or less mild agents and by dilution have been studied during recent years, especially by ultracentrifuge methods [Pedersen (1, 2, 3); Steinhardt; Lundgren; Williams & Watson], also by osmotic pressure [Burk (1, 2, 3), Ogston (1)] and solubility methods (see, for example, Sørensen & Palmer). The sedimentation studies of these phenomena have demonstrated that the dissociation reactions frequently follow simple multiple laws:

the first step in dissociation is mostly a splitting into halves, the first stage in association the doubling of the mass. Amino compounds are particularly effective as dissociating agents, for example urea, guanidine and its derivatives, also proteins and protein-like substances like clupein. The mutual interaction of proteins is particularly marked in serum, as first discovered by McFarlane [cf. *Ann. Rev. Biochem.*, 5, 142-144 (1936)] and studied in great detail by Pedersen (1, 2, 3), who has demonstrated that the phenomenon is quite frequent, and sometimes very striking. It must be taken into account in quantitative estimations of the amounts of the components in a mixture, as calculated from ultracentrifuge diagrams. Steinhardt, whose investigations on dissociation of proteins started from earlier observations on the marked effects on solubilities of proteins by various amino compounds, concludes from ultracentrifugal and diffusion measurements that in high concentration of urea and other amides hemoglobin is totally dissociated into half molecules, whereas the same amide concentrations do not change the molecular weight of pepsin. The effect is, in part at least, reversible. Spectroscopic tests, as well as gas-affinity and gas-capacity measurements, show no difference between the dissociated and the normal protein, which therefore must be nearly identical in their chemical properties. This bears a certain analogy to the fact that electrochemically no difference is found between split products and original molecules in the dissociation of the hemocyanins (Tiselius & Horsfall) and other proteins. Steinhardt explains the effect of amides by a weakening of polar association forces between adjacent peptide chains in the molecule through competition with the added amino groups. A similar theory for explanation of reversible dissociation phenomena is given by Wrinch (3) (cf. also p. 176). It is probable that other causes may also be operative, especially in view of the recent results of Pedersen. In his latest contribution (Royal Society discussion, Nov. 1938, as quoted by McFarlane) Pedersen suggests that the primary units of the structure (which may consist of polypeptide chains, possibly arranged in cyclol cages, as postulated by Wrinch, or in some other kind of units) are kept together with a non-protein "cement," *e.g.*, carbohydrates, phosphatides, and nucleic acids, which plays a fundamental rôle in the dissociation reactions. One protein may partially remove the "cement" from another, and thus influence its stability. The disulphide linkages in keratin, although probably stronger, form a somewhat analogous case, which has recently been studied further by Elsworth &

Phillips. Sreenivasaya & Pirie were able to disrupt the tobacco-mosaic-virus molecule into an unstable protein of low molecular weight and free nucleic acid by addition of sodium dodecyl sulphate. As nucleic acid seems to be present in all virus proteins, this effect may be of general importance. In this connection reference should be made to the interesting work on the structural rôle of the nucleic acid in chromosomes [see for example Astbury & Bell; W. J. Schmidt (1, 2); Caspersson; Caspersson & Schultz] and of the lipoids in the retinal rods [W. J. Schmidt (3)].

Many new papers which deal with molecular weight determinations and with the characterisation of proteins and enzyme proteins by molecular weight analysis have appeared (for work on the viruses see p. 169). Sumner, Gralén & Eriksson-Quensel (1, 2, 3) studied the crystalline proteins, urease, canavalin, concanavalin A, and concanavalin B, from the jack bean. Crystalline beef catalase was studied by Sumner & Gralén (1, 2); the molecular weight was found to be 248,000. Horse liver catalase was purified and investigated by Stern & Wyckoff (1, 2) and by Agner; the latter found the molecular weight to be 225,000. Gralén & Svedberg found the crystalline snake-venom protein, crotoxin, prepared by Slotta & Fraenkel-Conrat (1, 4, 5), to be homogeneous with a molecular weight of 30,000. The decrease in toxicity on reduction with cysteine, as found by Slotta & Fraenkel-Conrat (2, 3, 6), is accompanied by the formation of low molecular weight material, in agreement with the view that the molecule is split by dissolution of disulphide bonds. Sanigar, Krejci & Kraemer have studied gelatin, and Quensel & Svedberg the proteins in barley. Kabat & Pedersen, continuing the sedimentation studies of Heidelberger & Pedersen on horse and rabbit antibodies, have now also determined the diffusion constants, which has made possible the calculation of molecular weight. They have extended the work to a number of other animal species. The results show two definite groups of molecular weights, one of about 910,000 to 930,000 for antibodies obtained from the pig, cow, and horse, another of about 157,000 for the rabbit and monkey. Seibert, Pedersen & Tiselius have studied the molecular size and other physical properties of tuberculin and their relation to its biological properties.

Several papers which deal with improvements in the construction of ultracentrifuges have appeared. Boestad, Pedersen & Svedberg give a survey of the constructional development of the oil-turbine type and describe some recent improvements which have reduced the

costs of operation to about one third of the original value. The air-driven ultracentrifuge, based upon the spinning top of Henriot & Huguenard and developed during the last few years especially by Beams & Pickels, Bauer & Pickels, and Wyckoff & Lagsden, has become a very useful tool for measurements as well as for preparative work, particularly in the field of viruses. Beams, Linke & Sommer describe an improved vacuum type air-driven centrifuge especially suitable for spinning large rotors at constant temperature. Beams also describes a "tubular" vacuum-type centrifuge for continuous flow, which should be very useful for preparative work. Tiselius, Pedersen & Svedberg have constructed a new cell for the oil-turbine ultracentrifuge, in which a partition perforated with a large number of holes and covered with a hardened filter paper enables the cell content to be divided into two parts after concluding an experiment. Hughes, Pickels & Horsfall describe a new sampling device for high-speed concentration centrifuges, consisting of a hollow, valved plunger. Two papers of Pickels (1, 2) deal with new speed-measuring devices and an important improvement in the type of air bearings. Philpot has worked out a method of observation of sedimentation, which allows the concentration-gradient curves to be observed and photographed directly.

Whether sedimentation-equilibrium measurements or a combination of sedimentation-velocity and diffusion determinations (which is now the most common procedure) are used for calculation of the molecular weight, the frictional constant  $f$  is eliminated. The constant  $f$  is obtained from the diffusion constant  $D$  by the equation  $f = RT/D$  ( $R$  the gas constant, and  $T$  the absolute temperature) and can also be calculated from the molecular weight on the assumption that the molecule is spherical and non-hydrated; this value is usually designated  $f_0$ . In many cases good agreement is found between the two values for  $f$  thus obtained, which is usually taken as an indication that the molecule does not deviate appreciably from a spherical shape. In other cases, for example in antibodies and in tuberculin, the ratio  $f/f_0$  is much greater than 1.0, which indicates a non-spherical shape or a hydrated molecule. From the value of  $f/f_0$  axial ratios may be calculated according to the methods of Gans, of Herzog, Illig & Kudar, and of Perrin, as has been done for example by Polson. This procedure, however, is not very accurate.

Studies of particle shape, and of properties depending on shape, have always formed an important branch of colloid chemistry. A number of methods depending upon molecular orientation have been

worked out, of which the stream double refraction seems most promising, at least for protein solutions. Some years ago von Muralt & Edsall studied myosin, and Boehm & Signer ovalbumin, myogen, gelatin, myosin and ovoglobulin. The results indicated thread-like molecules for myosin and ovoglobulin, whereas ovalbumin and myogen showed no assymetry. Boeder, Haller, and Kuhn have recently improved the theoretical treatment, and the method is likely to become of great importance. Under suitable conditions the length of the molecule may be calculated from determinations of the position of the extinction angle at different rates of flow; observations of the double refraction at different velocity gradients make possible the calculation of the double refraction of the molecule itself. Takahashi & Rawlins (1, 2) found strong stream double refraction in the sap of plants infected with tobacco-mosaic disease and later in the purified virus protein also (3). A number of other viruses, studied by Bawden & Pirie (1, 2, 3) and Lauffer & Stanley, gave strong effects. The latter authors found weak but observable effects in hemocyanin solutions of *Helix* and *Limulus*, and of hog thyroglobulin, whereas the elementary bodies of vaccinia and the Shope papilloma-virus protein showed no effect, thus indicating a symmetrical shape. The spontaneous double refraction developed in certain concentrated virus solutions, as first observed by Bawden, Pirie, Bernal & Fankuchen, is a related phenomenon. The stream double refraction of sodium caseinate solutions and the influence of salts on the effect have been studied by Nitschmann. Lauffer (1) and Lauffer & Stanley describe a sensitive apparatus mainly intended for qualitative observations. Björnsthål & Snellman in the Upsala laboratory have recently worked out a much improved arrangement for quantitative measurements (unpublished). They have found certain antibody proteins to have a very elongated shape. Frampton & Neurath and Lauffer (2, 3), using Kuhn's equation for the viscosity of a colloidal solution, found the ratio of the long to the short axis for the molecule of the tobacco-mosaic virus to be 36.8 to 35.0, but it is emphasized that the assumptions underlying the equation are not necessarily valid in this case.

According to Polson, an equation of Kuhn's type but with somewhat different empirically determined constants is valid for a large number of different proteins, the axial ratios being calculated from the  $f/f_0$  ratios referred to above (p. 160). By the converse procedure molecular weights may be calculated from diffusion and viscosity

data [cf. also Svedberg (3)]. A number of other methods have been used for determining the shape of protein molecules, such as light-scattering measurements by Putzeys & Brosteaux and by Wöhlisch and collaborators, x-ray analysis, especially by Bernal & collaborators (see p. 156), and dielectric measurements (see below).

Studies of molecular weights by osmotic pressure have been published by Adair & Adair on hemoglobin, and by Burk (3) on gliadin.

#### ELECTROCHEMICAL PROPERTIES OF PROTEINS

Dielectric measurements, that is determination of dipole moments and relaxation times, form an important method for determining the electrical symmetry and the shape of protein molecules [cf. *Ann. Rev. Biochem.*, 4, 108-111 (1935)]. The experimental methods and the theoretical interpretation still offer some difficulties. Oncley obtained a dipole moment of about 500 Debye units for horse carboxyhemoglobin. Arrhenius found a much lower value, 17.8 to 41.6 Debye units, for hog carboxyhemoglobin, and from the time of relaxation calculated the axial dimensions for the molecule; his findings were in satisfactory agreement with x-ray measurements on methemoglobin by Bernal, Fankuchen & Perutz. It is possible, of course, that the horse and swine hemoglobins really are quite different. Ferry & Oncley found the increments in dielectric constant for the most soluble serumalbumin fractions greater than for the less soluble; for pseudoglobulin the increment was still greater. The relaxation times were considerably greater than those calculated from the molecular weights.

The relation between electrochemical properties and solubility in the presence of salts has been studied by Ferry, Cohn & Newman for carboxyhemoglobin, and by Cohn, McMeekin & Blanchard for cystine. Cannan reports an extensive series of careful measurements of the influence of salts on the titration curve of ovalbumin and Palmer's lactoglobulin. It was found that the slope of the dissociation curve of egg albumin on the acid side of the isoionic point, after correction for non-carboxylic groups, is dependent on the ionic strength only, without indication of specific ionic effects even at ionic strengths approaching unity. The isoionic point increased from 4.85 to close to 5.1 in 2*M* KCl (cf. the effect on the isoelectric point, below). Cohn discusses the recent results as to the relation of basic and acidic groups in proteins to the constituent amino acids by correlation of titration data with amino acid analysis, and gives a valuable survey of the results obtained by dielectric measurements.



Electrophoresis methods have become of rapidly growing importance in protein chemistry for electrochemical characterisation by mobility and isoelectric point determinations, for homogeneity studies and for preparative separations when drastic methods must be avoided. For recent summaries see Theorell and Tiselius (3, 4). The improved method of Tiselius (1) makes possible measurements by a convenient observation of moving boundaries with the *Schlieren* method as well as by chemical analysis. The differently migrating components of a mixture may be observed as lines in a spectrum and, with the aid of such observations, one may arrange the experiment to give maximum separation. In a recent paper by Tiselius (4) a number of different applications of this procedure are described, as well as micro- and macro-modifications of the apparatus. It has been possible by this method to prove that four distinct groups of proteins are present in normal sera [Tiselius (2)] and that in certain antisera a fifth protein component appears, which carries the antibody function (Tiselius & Kabat), and also to isolate the fractions. Landsteiner, Longworth & van der Scheer were able to differentiate egg albumins from different groups of birds (Anseriformes and Galliformes) by the same method; mixtures of both gave rise to two distinct boundaries. Adair (unpublished work in the Upsala laboratory) similarly could prove that fetal and maternal hemoglobin from sheep are quite different [cf. also *Ann. Rev. Biochem.*, 5, 465 (1936)]. Hesselvik has made an electrophoretic investigation on ovomucoid, and McFarlane & Kekwick on "bushy stunt" virus protein. Tiselius & Horsfall were able to demonstrate electrophoretically the cross combination of hemocyanins from related snail species after reversible dissociation of their mixtures. Seibert, Pedersen & Tiselius used the method for characterisation of tuberculin-protein preparations and for their separation from carbohydrate. Tiselius, Henschen & Svensson studied solutions of crystalline pepsin [cf. also *Ann. Rev. Biochem.*, 7, 40 (1937)] which were found to contain one electrochemically homogeneous protein component, which carried the peptic activity and was negatively charged even at pH 1; besides, however, considerable amounts of inert material of different migration velocity was present. By electrophoretic separation this could be removed and pepsin preparations with activity 31 to 69 per cent higher than the original value were obtained.

Measurements of electrophoretic mobility as well as of membrane potentials—the latter method especially used by Adair & Adair [cf.



*Ann. Rev. Biochem.*, 6, 163–169 (1937)]—give results which depend on the net charge of the protein molecule, whether or not this is due only to acid-base dissociation, as studied by titration.

Migration measurements on protein-coated collodion or quartz particles often give results which agree well with those obtained by the direct methods applied on proteins in solution. This has again been verified by Moyer (1, 2) on serum albumin and pseudoglobulin. On the other hand Abramson & Moyer found the isoelectric points of finely ground crystals of tyrosine, cystine, and aspartic acid near pH 2.3 although the isoelectric points of the dissolved acids are widely different. The discrepancy between the results obtained by Smith [cf. *Ann. Rev. Biochem.*, 6, 166 (1937)] using the microscopic method on egg albumin-coated particles and those obtained by Tiselius on dissolved egg albumin (which have been confirmed repeatedly, also using the new optical observation method) has been verified by Moyer (2), who found the isoelectric point to be pH 4.82 in 0.02 *M* acetate buffer, in agreement with Smith's value of pH 4.83, as compared to Tiselius' value of pH 4.55. The difference indicates that this protein suffers a change in its properties on adsorption—it is known that egg albumin is easily denatured at surfaces. Evidently one must be careful when drawing conclusions regarding the electrophoresis behaviour of dissolved proteins from measurements on coated particles, even though in most cases the two methods apparently give concordant results.

The possibilities of applying the Debye-Hückel theory to the electrochemical behaviour of proteins continues to attract the attention of those studying titration curves, membrane potentials, and electrophoretic mobilities. Moyer & Abels and Moyer & Abramson, by applying modified (empirically) Gouy-Debye-Hückel equation, were able to calculate titration curves, molecular weights and radii for egg albumin, phycoerythrin, horse-serum albumin and pseudoglobulin, from mobility data. As the difficulties in using the direct electrophoresis method over a wide range of ionic strengths have now been greatly reduced, Tiselius & Svensson in the Upsala laboratory have made investigations on the influence of salts on the mobilities of a number of well-defined proteins of varying molecular sizes (not yet published). The change of mobility with ionic strength for egg albumin at pH 7 follows the Debye-Hückel theory for the lower ionic concentrations, and its isoelectric point is lowered 0.12 pH units when the ionic strength increases from 0.01 to 0.10. A similar shift

of the isoelectric point of coated particles has been observed by Smith and Abramson & Moyer in the papers referred to above. The salt effect on the isoionic point is of opposite sign (see also Cannan, p. 162). These results emphasize again the necessity of distinguishing between isoelectric and isoionic conditions as well as of accurate data for the composition of the ionic medium when electrochemical properties are used for the characterisation of proteins.

#### ISOLATIONS AND PURIFICATIONS

The importance of using methods as gentle as possible in preparative work with proteins is now generally realised. The difficulties in protein preparation have always been closely connected with the difficulties in characterisation of these substances. Ultracentrifugal sedimentation and electrophoresis methods have increased our possibilities of detecting irreversible changes produced by too drastic treatment of the material, and have shown repeatedly that the preparations obtained by gentle methods usually are the most homogeneous and reproducible in molecular size and other physical properties. Work on the isolation of a number of biologically active substances as well-defined proteins has also emphasized this point, the specific activity being a particularly sensitive criterion of changes in properties dependent upon operations of a too drastic character.

Crystallisation is usually considered to be the final step in the purification and isolation of a protein. Kunitz & Northrop in an interesting paper discuss the solubility of crystalline proteins as a test of purity in connection with determinations on chymotrypsin and chymotrypsinogen. The latter possessed a solubility which was independent of the amount of solid phase; this indicates homogeneity, chymotrypsin gave evidence of the presence of a second component. The authors point out that almost all proteins so far examined have a solubility which varies more or less with the quantity of solid phase. This again calls attention to the fact that for most proteins it is by no means proved that they are chemical individuals. The high degree of homogeneity in ultracentrifugal sedimentation shown by many proteins does not necessarily prove a chemical homogeneity, since widely different proteins may have very nearly the same molecular weight. A tendency to form mixed crystals is also known to be great for many proteins.

As already emphasized a number of physical methods have frequently proved of value in preparative protein chemistry, such as ultra-

centrifugation, ultrafiltration (both especially suitable for viruses; see p. 169), electrophoresis, and adsorption. Separations which are difficult by crystallisation may be easily performed by electrophoresis and vice versa. In judging the possibilities and results obtainable by these methods (or at least the first three of them) one must remember that these separation processes are too gentle to break down complexes in solution, if these complexes are not already appreciably dissociated into their components. This may be a drawback in some cases, for example if we are looking for the ultimate chemical individuals which constitute a system; in such a case one may have to proceed with more drastic methods. If, however, we seek information regarding the components of a system, especially as they occur in the native condition in biological fluids the first mentioned or other similarly gentle methods are most likely to be useful. Recently, the relative merits of different isolation methods for virus proteins has been the subject of an interesting discussion (see p. 170).

Attempts to isolate chlorophyll-protein compounds, which are assumed to exist in plant cells and to play an essential rôle in photosynthesis, have been published by E. L. Smith, and similar investigations on the chromoproteins of synthetic purple bacteria by French. Other examples of attempts to isolate proteins and protein complexes with other substances from cells in as nearly a native state as possible are several contributions on proteins and nucleoproteins from bacteria, for example by Menzel & Heidelberger, Chambers & Weil, Thompson & Dubos, Sartory, Sartory & Meyer, Sevag, Lackman & Smolens, and Seibert, Pedersen & Tiselius. Ovoverdin, a carotenoid-protein pigment showing certain analogies to visual purple has been isolated from the egg of the lobster by Stern & Salomon, and by Kuhn & Sørensen, who have identified it as a protein compound of astaxanthin, a xanthophyll also found in the shell of the lobster.

Mann & Keilin obtained from red blood corpuscles a crystalline copper-containing protein, for which the name hemocuprein is suggested. Dalton & Nelson isolated from aqueous extracts of a wild mushroom (*Lactarius piperatus*) a crystalline copper-containing protein possessing tyrosinase activity. Agner, in the purification of catalase, obtained a copper-containing protein which possibly is an integral part of the catalase system. Kubowitz (1, 2) was able to split off reversibly the copper constituents of hemocyanin and polyphenoloxidase.

Cole has prepared a number of crystalline ovalbumins which were characterised by their pepitin reactions. Pillemer & Ecker have

reported some interesting experiments on the specificity of human hair, wool, and chicken keratins, which have been made accessible for experiments of this kind by application of the reduction method of Goddard & Michaelis to bring them into solution.

Many interesting proteins of biological activity have been studied, some of which are referred to in other chapters of this volume. Northrop describes the concentration and purification of a nucleoprotein with the properties of bacteriophage from lysed staphylococci cultures. The rates of diffusion and sedimentation of this protein are the same as those of the active agent, and its inactivation by heat, acid and chymotrypsin digestion is roughly proportional to the amount of denatured protein formed. An ultracentrifugal analysis made by Wyck-off (2) indicates a particle weight of 300 to 500 millions. Slotta & Fraenkel-Conrat (1 to 6) were able to isolate two active proteins from rattlesnake venom, one of which "crotoxin," represents the neurotoxic principle and was obtained in crystalline form (see also p. 159). Snake-venom proteins have also been isolated and studied (especially regarding the rôle of sulphur) by Micheel & Schmitz (1, 2) and Micheel & Bode (1, 2). Walti has crystallised the papain-like proteolytic enzyme ficin, and Herriott (1, 2) swine pepsinogen. By transformation of this protein into swine pepsin by chicken pepsin Herriott, Bartz & Northrop could demonstrate that the species specificity is present in the precursor. Tiselius, Henschen & Svensson have purified crystallised pepsin by electrophoresis. Du Vigneaud, Irving, Dyer & Sealock and Irving & du Vigneaud have demonstrated that the pressor and oxytocic hormones in untreated press-juice of the posterior lobe of the pituitary gland have different electrophoretic migration velocities; this fact is made use of for their separation.

Bondy & Freundlich obtained two proteins with different isoelectric points from preserved *Hevea* latex, and Luck has studied the conditions for extraction and fractionation of liver proteins.

The serum proteins continue to attract much attention. A number of different methods have been used for isolation and characterisation of the various components: solubility, ultracentrifugation, electrophoresis, and immunological reactions. Hewitt has continued his investigations in which especially the carbohydrate content of the protein fractions serves as a guide and has reported on the preparation and the properties of a globulin present in the albumin fraction (1). Hewitt also gives a summary of his recent work together with new results (2). Kekwick, by salting out with sodium sulphate, obtained

two crystalline fractions from horse-serum albumin, of which one was free from carbohydrate and seems to be nearly identical with Hewitt's crystalalbumin; the other was rich in carbohydrate (1.95 per cent), but contained less than was found by Hewitt in his seroglycoid (amorphous). Green, using isoelectric precipitation, finds three different globulin fractions; this in agreement with the results obtained by Tiselius (2) (see p. 163) by electrophoresis. Kendall, who has used especially immunological means for identifying globulin fractions, made the interesting observation that two soluble fractions may give an "euglobulin" precipitate on mixing, which is also reported by Hewitt (1, 2) for his fractions, globoglycoid and serumglobulin A. Jameson & Roberts make use of phase-rule considerations in the characterisation of serum globulins and have also obtained evidence of the existence of definite components.

In addition to the electrophoretic studies referred to above (p. 163), mention should be made of an investigation by Stenhagen who was able to demonstrate the existence of fibrinogen in plasma as a protein component migrating between the  $\beta$ - and  $\gamma$ -globulin fractions. The earlier findings of Kylin & Paulsen (cf. *Ann. Rev. Biochem.*, 6, 165 (1937)) regarding the existence of a fibrinogen with an isoelectric point of pH 12.4 could not be confirmed. Stenhagen & Teorell have made interesting observations on the displacement of bilirubin from its complex with serum albumin by addition of nucleic acid. Holiday & Ogston, using ultraviolet absorption and titration curves, found antibodies to Types I and II pneumococcus to be different from each other and from normal serum globulins; they also found Hewitt's seroglycoid to be more like globulin than albumin in its properties as studied by these methods.

From ultracentrifugal studies Svedberg & Andersson conclude that sera from mammals, birds, reptiles, amphibians, and fish are similar with regard to molecular state, whereas those from the Cyclostomata differ considerably; this agrees with an earlier comparison between the molecular weights of the respiratory proteins for these classes.

In the reviewer's opinion there is now hardly any reason to doubt the existence of distinctly different protein components in native sera, mainly because of the evidence given by methods as gentle as ultracentrifugation and electrophoresis [cf. the "orosin" theory of R. J. Block (1)]. On the other hand it seems quite likely, especially for the globulins, that each component is not a single chemical individual but rather a group of substances of similar physicochemical properties,

which are distinctly different from those of the other components. No doubt there is a certain interaction between the components already in the native serum, which is evident especially from the mutual influence of serum proteins on the molecular size of each, as studied by McFarlane and by Pedersen (see p. 158). It is, however, extremely difficult at the moment to correlate the results found by different fractionation methods, based upon different properties of the system. When the interrelations between the fractions obtained by a number of varied but not too drastic methods have been established we will probably be able to obtain a much clearer picture of the constitution of this complicated system.

#### THE VIRUS PROTEINS

A number of valuable surveys over this rapidly developing field have appeared, for example by Stanley (1 to 5) and by Lynen. Reference can be made here to only a few papers of chemical interest. Best and Loring (1) have confirmed the observation that tobacco-mosaic virus is a nucleoprotein. Bawden & Pirie (4) isolated from tomato plants infected with "bushy stunt" a virus nucleoprotein in a fully crystalline state; it formed beautiful dodecahedra belonging to the cubic system. This is important, as the earlier isolated virus crystals were paracrystalline with only two-dimensional regularity (Bernal & Fankuchen). Its physical properties were studied by McFarlane & Kekwick, who found it homogeneous in sedimentation and electrophoresis. Sedimentation equilibrium gave a molecular weight of 8,800,000, in good agreement with the value calculated from sedimentation velocity on the assumption of a spherical shape, with a radius of 13.7  $\mu$ . X-ray measurements by Bernal, Fankuchen & Riley showed the crystals to be built of a body-centered cubic lattice of side 394 Å, containing two particles per cell.

As the tobacco-mosaic and related virus protein molecules are very elongated and until recently no accurate diffusion measurements were available there has been considerable uncertainty regarding their molecular weights, although the sedimentation constants are known accurately. Frampton & Neurath and Lauffer (2, 3), from their determinations of the axial ratios referred to above (p. 161) and by combination with x-ray data or sedimentation constants, arrived at a molecular weight for the tobacco-mosaic virus of 40 to 100 millions. Recently, Neurath & Saum, using the method of Lamm (1, 2), obtained a diffusion constant of  $3 \times 10^{-8}$  sq. cm. per sec. for dilute solutions; with the sedimentation constant previously determined this

would correspond to a molecular weight of 60 millions, and an axial ratio of 1:55.

Thornberry describes an improved crystallisation method for the tobacco-mosaic virus. A chemical and optical study of potato virus "X" has been published by Bawden & Pirie (2). Ultracentrifugal isolation and analysis have been made by Price & Wyckoff on the cucumber viruses 3 and 4 which were found to be similar to the tobacco-mosaic proteins. A substance of high molecular weight other than the virus protein was also present. Loring, Osborn & Wyckoff were able to isolate proteins of high molecular weight from (healthy) broad bean and pea plants. Bawden & Pirie (3) similarly obtained heavy proteins from normal tobacco and tomato leaves. They were not nucleoproteins, and serologically were not related to the virus of infected plants—thus there is no reason to believe that they are "precursors" to the virus.

The aucuba-mosaic virus and the pH-stability regions of tobacco-mosaic virus and papilloma-virus proteins were studied by Wyckoff (1, 3), and Beard & Wyckoff. In both cases there is a parallelism between loss of activity and disintegration of the particles at the end of the stability zone, indicating that the infectivity is a property of the protein molecules. In slightly alkaline solutions of the papilloma virus, however, there is a gradual loss of activity which is not accompanied by a change in sedimentation. Compare also the earlier observations on the tobacco-mosaic virus by Stanley (6), according to which hydrogen peroxide, formaldehyde, nitrous acid, and ultraviolet light led to inactivation without any change in sedimentation behaviour, serological properties, or crystallisability. Recently Ross & Stanley, in a more detailed study, found that the interaction with formaldehyde led to a simultaneous loss in activity and a reduction in the number of free amino- and tyrosine groups. The activity could be partially restored by dialysis at pH 3. Evidently these groups are essential for the activity of the virus.

The possibility that purification methods may lead to the aggregation of virus particles has been discussed by Bawden & Pirie (5) who emphasize the need for caution when interpreting centrifugal data on systems which can easily aggregate under suitable circumstances. Loring, Lauffer & Stanley, and Loring (2), on the other hand, consider that only preparations obtained by rapid chemical treatment in the cold or preferably by ultracentrifugation in the cold are comparable to the virus in the untreated juice. The high degree of homogeneity shown by these preparations in sedimentation seems to support

the view that their particle size, whether they are aggregates or not, is a fundamental property. Compare also the work on the dissociation of proteins referred to above (p. 157) and especially the work of Sreenivasaya & Pirie. For investigations of the shape of virus molecules see also above (p. 161).

Attention is called to a number of valuable contributions to the discussion on virus research arranged by the Royal Society and published in *Proc. Roy. Soc. (London)*, B, 125, 291 (1938). See also *Nature*, 141, 564 (1938).

### CHEMICAL ANALYSIS

A number of new amino acid analyses on proteins give further support to the Bergmann-Niemann periodicity rule [cf. *Ann. Rev. Biochem.*, 7, 110-111 (1938)]. Thus silk fibroin was analysed by Bergmann & Niemann, and elastin by Stein & Miller. According to Astbury & Bell wool keratin also conforms to the rule. The variable sulphur content of keratins introduces certain difficulties. Cow- and chimpanzee-hair keratins were analysed by W. D. Block & Lewis. The molecular ratio histidine:lysine:arginine was approximately 1:4:10, which is comparable to the value 1:4:12 proposed by R. J. Block & Vickery in their characterisation of keratins [cf. also R. J. Block (2)]. Vickery has published new determinations of histidine and arginine in zein—a protein that is remarkably simple. Some difficulties in the periodicity rule have been emphasized by Astbury (1), who points out that in some cases the calculated frequencies would require that one and the same place in the polypeptide chain be occupied by two or more amino acid residues. Svedberg (3) calls attention to the fact that hemoglobin, although normally of molecular weight 69,000, can be split reversibly into halves of molecular weight 34,500 (see also p. 157), which are probably chemically equal, whereas Bergmann & Niemann from cystine analysis, calculate a minimum molecular weight of 69,000. Bergmann & Niemann point out that deviations from the rule are to be expected for protein molecules which split reversibly into chemically different components. A review by Niemann gives a valuable summary of recent results in this important field. Interesting comparative studies of the amino acid composition of keratins, hemoglobins, neuroproteins, serum proteins, and neurokeratins of different animals have been published by R. J. Block (2). Comparative analyses of respiratory proteins from various animal classes have been made by Roche & Fontaine. Special interest has



been given to determinations of sulphur groups in proteins, which frequently play an important rôle in their biological activity and structure. Reference should also be made to the work on snake-venom proteins by Slotta & Fraenkel-Conrat (1 to 5), Micheel & Bode (1, 2), and Micheel & Schmitz (1, 2), and to the work on casein, lactalbumin, edestin, and papain by Kassell & Brand (4). Greenstein, using the method of Kuhn & Desnuelle (see below), studied the liberation of sulphhydryl groups which takes place when urea and guanidine derivatives act on egg albumin (cf. p. 158). Possibly sulphhydryl groups play an important rôle in the structure of this and other proteins, even if one must admit that other groups may also be liberated, which have escaped our attention on account of the lack of suitable analytical methods. Kuhn & Desnuelle show that the strongly reducing dye porphyrindine can be used for determination of sulphhydryl groups at room temperature, which makes it better suited for investigations on native proteins than earlier methods of a similar kind, which required high temperatures, and easily led to denaturation.

Analytical studies of carbohydrate constituents and investigations of the rôle of carbohydrates in the protein molecule (especially in relation to immunological specificity) continue to attract much attention. Reference has already been made to work on serum proteins from this aspect (see p. 168). For a recent review see Meyer. The glucosamine method of Elson & Morgan and Nilsson has been further improved by Sørensen. Neuburger (2), applying a method based upon tryptic hydrolysis followed by acetylation of the split products with ketene and chloroform extraction, was able to isolate a carbohydrate component from crystalline egg albumin with a molecular weight of about 1200; it probably contained four mols of mannose and two mols of glucosamine, together with an unidentified nitrogenous constituent. Ogston (2) has made an attempt to remove the bound carbohydrate from serum pseudoglobulin with a commercial enzyme preparation. Hewitt (3) analysed a number of proteins and their digestion products for carbohydrate constituents, and investigated their reducing power, which is also attributable to certain of the amino acids.

For work on lipoproteins see Chargaff; Wagner-Jauregg & Arnold; and Macheboeuf & Tayeau. For nucleoproteins see the virus, bacteriophage, and bacterial protein papers referred to above, as well as recent work on prosthetic groups in some enzymes (cf. p. 167). Chemical modification of biologically active proteins by suitable methods offers a means of localizing the specific action to certain groups in the molecule. Acetylation with ketene continues to be used fre-

quently; thus Stern & White found that acetylation of the tyrosine residues (hydroxyl group) of insulin reduces its activity, which is not the case for the amino groups; this is analogous to the earlier results of Herriott & Northrop for pepsin. Pappenheimer similarly studied the action of ketene and formaldehyde on diphtheria toxin. Papers by Neuberger (1) on the action of acetylating agents on amino acids and by Jackson & Cahill on racemization of amino acids on ketene treatment are valuable for the interpretation of these and similar experiments. Philpot & Small have studied the action of nitrous acid on pepsin. Clutton, Harington & Yuill (1, 2) have continued their investigations of the immunochemistry of synthetically modified proteins. For these contributions the reader is referred to the chapters on proteolytic enzymes and immunochemistry.

#### AMINO ACIDS

Reference can be made only to a few representative papers. Kurtz succeeded in a simple synthesis of *dl*-citrulline from *dl*-ornithine. Shemin & Herbst, and Herbst describe the synthesis of dipeptides from  $\alpha$ -ketoacids. Patterson & du Vigneaud have prepared tetra-deuterothomocystine and dideuteromethionine. Freudenberg & Keller have synthesised amino acid esters of choline, which are of importance in relation to the structure of the oxytocic hormones.

The valuable method introduced by Van Slyke & Dillon of determining carboxyl groups gasometrically by treatment with ninhydrin [cf. *Ann. Rev. Biochem.*, 7, 105 (1938)] is described in detail in a new paper by the same authors. The reactions between ninhydrin, alloxan, and isatin with amino acids and polypeptides have been investigated by Abderhalden. Virtanen & Laine utilize the aldehyde formed in the ninhydrin reaction for determining amino acids. A valuable summary of methods for amino acid determination has been published by R. J. Block (3). Bergmann in a new paper reports on his recent work in developing reagents for precipitating aliphatic mono-amino monocarboxylic acids. Stein, Niemann & Bergmann describe an interesting method of estimating individual amino acids in protein hydrolysates by means of the solubility product. The method seems capable of wide application when selective precipitation reagents only cause incomplete precipitation, as is mostly the case. Titration of acidic and basic groups in the presence of dioxane is described by Popovici & Radulescu, and the perchloric-acetic acid method by Toenies & Callan. New methods for alanine and glycine are used by Bergmann & Niemann in their analysis of silk fibroin. Desnuelle and Froma-

geot & Heitz describe the determination of alanine and serine plus aspartic acid. Modified colorimetric methods for cystine and methionine are used by Kassell & Brand (1 to 4) and by Schöberl & Rambacher. Sullivan, Milone & Everitt report a rapid procedure for estimating the tryptophane content of casein, and Plimmer & Lowndes investigate the bromination method for this amino acid. Attempts to separate tyrosine, thyroxine, and 3,5-di-iodotyrosine by their different solubilities in acetic and butyric acids have been made by v. Przylecki & Truszkowski. The separation of arginine and histidine has been investigated by Mourot & Hoffer.

Edsall has published a review of his investigations on the Raman spectra of amino acids. Lindquist & C. L. A. Schmidt have measured dielectric constants for a number of amino acids, with special attention to the question of association in solution. Greenstein, Klemperer & Wyman have studied some physical properties of cystinyl peptides; their dielectric measurements indicate free rotation around single bonds in the peptides. Cohn, McMeekin & Blanchard have investigated the solubility of cystine in presence of salts and another dipolar ion. Solubility measurements have also been made by Dunn & Ross (water-ethyl alcohol mixtures) and by v. Przylecki & Kasprzyk-Czaykowska (in acetic and butyric acid). The interaction between amino acids and sugars has been further studied by Frankel & Katchalsky (cf. Balson & Lawson) and by Helferich & Mittag. The resolution of racemic amino acids into their optical antipodes through the salts of cholestenonesulphonic acid has been studied by Triem.

#### SURFACE CHEMISTRY OF PROTEINS

The method of Blodgett and of Langmuir, Schaefer & Wrinch of preparing "built-up" films of proteins on solid surfaces, consisting of a large number of oriented monolayers, has been used by Astbury, Bell, Gorter & van Ormondt for preparing films of egg albumin, containing up to 1764 monolayers. Mechanical, optical and x-ray investigations indicated that the monolayers consisted of polypeptide chains preferentially oriented in the direction of movement used in preparing the film, with their side chains more or less normal to the surface. From direct measurements of the thickness of the film a monolayer thickness of  $9.5 \pm 0.2$  Å was found; this is in good agreement with the side chain spacing determined from x-ray diagrams. It seems probable that these films were pre-formed on the surface of the solution from which they were prepared. These and other investigations on protein monolayers on surfaces indicate the close relationship be-

tween globular and fibrous structures. Contributions to the question of whether spreading involves denaturation have been made by Bull (1, 2) who has shown that it is possible to spread denatured egg albumin on a medium of suitable pH, and by Langmuir & Schaefer (1) who found that monolayers of urease and pepsin showed considerable enzymatic activity (cf. also Wu & Wang and Bull & Neurath). Langmuir & Schaefer (2) have produced a new type of protein film by a salting-out procedure. Seastone studied films of some high molecular proteins including tobacco-mosaic virus, and Harkins & Anderson films of oxidised cytochrome-*c*. Interaction in films between proteins and fatty acids was investigated by Neurath, and between antigens and antibodies by Shaffer & Dingle. Langmuir & Waugh have studied the adsorption of proteins at oil-water interfaces.

#### DENATURATION AND HYDRATION OF PROTEINS

Reviews of recent results in investigations of denaturation have been published by Bull (3) and by Mirsky (2). Wrinch (2) has discussed hydration and denaturation from the point of view of the cyclol theory. A considerable amount of water must be enclosed in the cyclol cages, but it is also assumed that the fabric forming the cages may be hydrated. In protein crystals a third kind of hydration in channels between molecules is postulated. It is, of course, extremely difficult to test these conceptions experimentally. Denaturation is supposed to depend upon a partial opening up or fragmentation of the cages, which would also take place in the formation of films. The cage-enclosed water must then be set free which is in agreement with the large difference in hydration found by Neurath & Bull for dissolved and surface-spread egg albumin. The hydration of proteins is also discussed by Jordan-Lloyd in a recent paper. Mirsky (1, 2), from an extensive investigation on the denaturation of myosin in relation to changes which take place in muscle, concludes that denaturation is probably related to muscle activity. Wöhlisch & Jühling and Jühling & Wöhlisch present evidence for regarding the coagulation of fibrinogen by thrombin as a kind of denaturation.

#### STRUCTURE OF PROTEINS

No doubt there is strong evidence that the polypeptide-chain theory is insufficient for explaining many properties of protein molecules, especially of the globular type; it must, therefore, be supplemented by additional assumptions as to the arrangement in space of the chain components, and of the forces acting between them. This is apparent

not only for certain physical properties, as for example those which depend upon the shape of the molecule, but also for the interpretation of the biochemical properties of many proteins. If the specific activity of an enzyme or virus depends ultimately only on a characteristic sequence of a large number of amino acid residues in a chain, it is extremely difficult to account for the inactivation by denaturation (frequently reversible) or by other comparatively gentle methods, which cannot be expected to bring about a rearrangement of this sequence. Both from physical and from biochemical aspects, therefore, a knowledge of the principles according to which the polypeptide chain is modified to form a protein molecule (and *vice versa*) is of paramount importance. A number of facts brought out by new and old studies in protein chemistry are of particular significance, as they indicate that there must be a comparatively simple and definite basis for these principles: x-ray diffraction patterns, the high degree of symmetry shown by protein crystals, the step-wise increase in molecular weight from one group of proteins to another, the periodicity in the sequence of amino acid residues, and the high degree of specificity shown by most proteins. The discussion of these structural problems has been particularly lively during the past year. A good picture of the state of affairs at present is obtained from the recent surveys by Astbury (2, 3), Astbury & Bell, and by Wrinch (5). Astbury thinks of the globular protein structure as a three-dimensional generalisation of the folds known to be formed in fibrous proteins like keratin and myosin. No longer is a sharp difference believed to exist between fibrous and globular structures, as the x-ray patterns of the former have been shown to contain certain features in common with the latter [Astbury (1)]. Wrinch (3) has developed further the cyclol theory with special reference to molecular weights (for Wrinch's theories of hydration and denaturation see p. 175). The most striking consequence of this theory is the explanation it affords of the step-wise grouping of molecular weights, as only  $72 n^2$  amino acid residues may be accommodated in the closed cyclol skeletons. It is pointed out (cf. also p. 156) that this law determines the number of groups only, and as their weights and the number of water molecules associated with the different groups under the conditions of molecular weight determinations are not accurately known, we cannot always expect an exact agreement with molecular weight data. The dissociation phenomena can be easily explained on the assumption of compound cyclol structures—the huge hemocyanin molecule would be a kind of “cage

colony." It is conceivable that such structures can be dissociated only in a step-wise manner, owing to the limited number of stable associations possible. The assumption of compound structures increases the number of possible molecular weights considerably: the 17,600 class would be explained as a combination of two  $C_1$  cyclols (each with 72 residues), and so on. The interlinking forces may be salt linkages or hydrogen and hydroxyl bonds, which would depend upon the acidity of the medium. This would account for the dependence of stability on pH. The capacity of water, urea and certain other molecules to form hydrogen and hydroxyl bonds with the protein fabric would explain the dissociation by dilution and by addition of various compounds referred to above (p. 158). Similar explanations of the dissociation phenomena have been suggested by other authors and need not, of course, necessarily be based upon the cyclol theory. Wrinch (1); Wrinch & Langmuir; and Langmuir & Wrinch have subjected the theory to further rigid tests by comparing its predictions with the results of the recent careful x-ray measurements of Crowfoot on crystalline insulin, which has a comparatively simple structure containing only one molecule per unit cell. They have found remarkably good agreement, although it still seems to be a matter of discussion as to what extent this agreement excludes other possibilities. The type of ring-chain tautomerism required for polymerisation of amino acids to a cyclol fabric appears to be in disagreement with chemical properties of simple substances of similar structure, as has again been pointed out by Haurowitz (1) and by Meyer & Hohenemser. From the chemical point of view one must also ask if there is only one or several "potential" polypeptide chains corresponding to a certain cyclol structure, that is, if the cage can be opened up in a unique way or not. This is particularly important from the point of view of the periodicity law found by Bergmann & Niemann (cf. p. 171). The connection between this stoichiometrical rule and the cyclol fabric and cage theory does not yet seem quite clear, even though it is evident that the hexagonal symmetry requirements in these models would favour the occurrence of the factors 2 and 3 in the periodicity rule. The step-wise grouping of molecular weights is, however, explained in an apparently independent way in both cases. The basis for the regularities found by Bergmann & Niemann is assumed to be a sequence of highly specific enzymatic synthesis reactions which, by successive operation, build up the polypeptide chain, whereas the limitation in the number of possible cyclol structures is based upon principles

of a purely geometrical nature. It is possible, as suggested especially by Astbury & Bell, that there is a common factor in the synthesis of all proteins, which when discovered will make it possible to define the relations between the different conceptions in a more definite way.

The cyclol theory explains surprisingly well a number of important properties of the proteins and has a much more precise form than alternative possibilities which have been discussed, at least with respect to the arrangement of the constituent groups in space. It is to be expected that the most decisive information for testing the theory will be obtained from further x-ray investigations on protein crystals.

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# THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF SULFUR

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*Synthetic studies.*—As an approach to a number of important biochemical problems, Greenstein has prepared and studied an interesting series of di- and tripeptides. In order to study the behavior of polythiol compounds upon oxidation, he synthesized (1), as a model of the more complex protein molecule, anhydro-*l*-cysteinyl-*l*-cysteine. *l*-Cysteinyl-*l*-cysteine ethyl ester hydroiodide (prepared by the coupling of dicarbobenzoxycystyl chloride with two molecules of cysteine ethyl ester in chloroform solution, followed by reduction with phosphonium iodide) was dissolved in ethanol saturated with ammonia at 0° which caused condensation to the piperazine ring and crystallization of anhydro-*l*-cysteinyl-*l*-cysteine. Upon oxidation with hydrogen peroxide in aqueous solution, the dimeric form, bisanhydro-*l*-cystinyl-*l*-cystine, appeared. Thus the smallest possible cyclic configuration was assumed, rather than extended polymeric forms. Greenstein also prepared, by the same general procedure, several diketopiperazines of cystine with the dicarboxylic amino acids and with tyrosine. The anhydro-*l*-cysteinyl-*l*-cysteine was further utilized as a starting point for the preparation of *l*-cystinyl-*l*-cystine (2). The piperazine ring was opened by hydrolysis by the slow action of concentrated hydrochloric acid and the resulting thiol derivative oxidized to the disulfide form. Later, Greenstein (3) synthesized the double tripeptide, *l*-cystinyldidiglycine, in order to study the action of various peptidases, thus providing a substrate which would be soluble enough to be used in high concentration and which would yield on hydrolysis an amino acid sufficiently insoluble to be filtered off and estimated.

As an approach to a study of the rôle of the  $\gamma$ -linkage in the chemical activity of glutathione, du Vigneaud, Loring & Miller synthesized isogluthathione,  $\alpha$ -glutamylcysteinylglycine. They utilized their previous observation that opening of carbobenzoxylglutamic anhydride with S-benzylcysteinylglycine in pyridine solution yields predominantly the derivative of the  $\alpha$ -peptide.

Following the researches of Schoenheimer and associates in sterol and fat chemistry, Patterson & du Vigneaud synthesized tetradeutero-homocystine- $\beta, \beta', \gamma, \gamma'$ - $d_4$  and dideuteromethionine- $\beta, \gamma$ - $d_2$ . Dideutero-

acetylene was prepared by the reaction of calcium carbide with deuterium oxide, reduced by chromous chloride and converted to the dibromide. The resulting dideuteroethylene bromide was condensed with benzyl mercaptan to form the dideuteroethylthioethyl bromide. The latter compound was converted to dideuteroethylhomocysteine by means of the malonic ester amino acid synthesis. From this compound tetradeuterohomocystine was prepared by reduction in liquid ammonia followed by oxidation; deuterohomomethionine was obtained by reduction and subsequent methylation. The extreme stability of these compounds in the presence of boiling hydrochloric acid has been demonstrated by Rittenberg *et al.*

Metallic sodium in liquid ammonia was introduced by du Vigneaud *et al.* (1930) as a reagent for the reduction of disulfide bonds. Later this reagent was employed in the reduction of other sulfur derivatives. It has again proved useful (Dyer) in the preparation of S-ethylhomocysteine (ethionine) by reduction of S-benzylhomocysteine and treatment of the reduced product with ethyl bromide. Jones & du Vigneaud prepared hexocystine by methods analogous to those employed for synthesis of pentocystine, and by reduction in liquid ammonia with subsequent addition of methyl iodide, hexomethionine was obtained. Brand, Block & Cahill (2) synthesized *l*-methyl-S-cysteine by the method of Clarke & Inouye (1931-32) by reduction of cystine with tin and hydrochloric acid followed by treatment with dimethylsulfate in alkaline solution.

Toennies (1) described the synthesis and properties of a mixed disulfide, S-(guanythio)-*l*-cysteine, which was produced by a reaction between the two disulfides, cystine and dithioformamidine (thiourea as catalyst), according to the following equations:  $R \cdot S \cdot S \cdot R + R' \cdot SH \rightarrow R \cdot S \cdot S \cdot R' + R \cdot SH$ ;  $R \cdot SH + R' \cdot S \cdot S \cdot R' \rightarrow R \cdot S \cdot S \cdot R' + R' \cdot SH$ . He presents evidence that it tends to hydrolyze into thiourea and cysteine sulfenic acid. The production of a mixed disulfide as well as of a method by which a highly reactive sulfenic acid may be procured in solution opens important possibilities in physiological studies. Toennies (3) has reported the production of a sulfoxide of methionine, which it is hoped will prove useful in studies of the intermediate metabolism of methionine.

For discussion of the outstanding work in the syntheses of co-carboxylase readers are referred to the chapter on non-proteolytic enzymes.<sup>1</sup>

<sup>1</sup> *Ann. Rev. Biochem.*, 8, 59 (1939).

*Physical chemistry.*—Borsook, Ellis & Huffman have derived from thermal data a value of  $-0.390$  volts for the normal oxidation-reduction potential of sulfhydryl at pH 7.0. They conclude that the higher values previously obtained by direct measurement for the cystine  $\rightleftharpoons$  cysteine system are not referable exclusively to this system.

As a preliminary to a systematic investigation of systems containing neutral salts and more than one protein, McMeekin *et al.* studied the solubility of cystine in water and in ethanol-water mixtures containing neutral salts. Empirical equations were developed for interaction between ions and dipolar ions which describe the shape of the solubility curve at all concentrations of the salts studied. They also (Cohn *et al.*) employed a system containing neutral salts, sodium chloride and sodium sulfate, glycine and cystine, and evolved an equation giving the solubility ratio of cystine in terms of "salting out" constants, related to the electric moments of cystine and to the ionic strength and dielectric constant of the solution.

The isoelectric point of the surface of crystalline cystine in hydrochloric acid and in acetate buffers of approximately constant ionic strength was found by Abramson & Moyer to be near pH 2.3 in all cases investigated. When suspended in gelatine, the crystals adsorb the protein and attain a surface identical in behavior with gelatine-coated quartz or collodion particles.

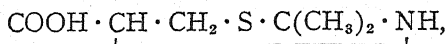
*Reactions and methods of determination.*—Ratner & Clarke investigated further the two classes of compounds described by Schubert (1935, 1936): the first, semimercaptals, formed by addition of a mercaptan and an aldehyde; and the second, those in which an amino group near the sulfhydryl permits condensation also, with formation of a thiazolidine ring. They studied in detail the reaction between formaldehyde and cysteine with formation of thiazolidine-4-carboxylic acid and that between formaldehyde and  $\beta$ -aminoethylmercaptan with production of thiazolidine. They determined the acidic and basic dissociation constants together with the effects of various substituents in the molecule and the stability of the ring under different conditions. This study possesses added importance in view of possible reactions of this type in biological fluids. Schubert reported that the reaction product of cysteine and pyruvic acid has the thiazolidine structure rather than an open-chain addition form as reported previously (Schubert, 1936).<sup>2</sup> He also supplied the amino group in a third molecule, so that instead of thiazolidines, substituted

<sup>2</sup> *Ann. Rev. Biochem.*, 6, 198 (1937).



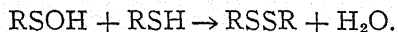
thio ethers, compounds of the type  $R \cdot CH \cdot (SR') \cdot NH \cdot R''$  and  $R \cdot CH \cdot (SR')NR''_2$  are formed.

Woodward & Schroeder isolated from acetone solution a condensation product of cysteine and acetone,

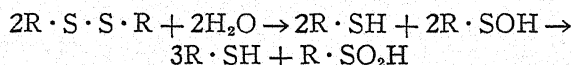


unstable in aqueous solution, being hydrolyzed into its original constituents. Because of this reaction the authors have suggested precautions in the application of the Linderstrøm-Lang acetone-hydrochloric acid method of amino acid titration. Todrick & Walker (2) have reported a compound formed by the addition of allyl isothiocyanate to the amino nitrogen of cysteine, with subsequent rearrangement of the isothiocyanate to the thiocyanate form. Morgan & Friedmann (1) isolated addition products of maleic acid with the three thiols, cysteine, thiolactic acid and glutathione and demonstrated that they are the general type,  $HOOC \cdot CH_2 \cdot CH(COOH) \cdot SR$ . In the case of thiolactic acid and glutathione, the excess maleic acid was simultaneously rearranged to fumaric. Kinetic studies indicated that addition and rearrangement proceed simultaneously, resulting in production of less than one equivalent of fumaric acid.

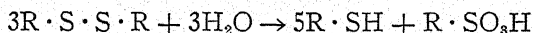
Toennies (2) tested the hypothesis that the oxidation of cysteine proceeds through the intermediate stage of a sulfenic acid as postulated by Matthews (1924) and others, rather than going directly to cystine. He oxidized cysteine perchlorate with permonosulfuric acid in isoamyl alcohol and obtained an immediate precipitation of oxidation products in the form of sulfates. By applying various analyses, Toennies found evidence that the main reaction product is a sulfate of oxycystine which reacts with cysteine to form cystine:



Evidence that the dismutation of cystine follows the path



instead of the previously suggested



has been presented by Lavine. Dismutation was effected by mixing sulfuric acid solutions of cystine and mercuric sulfate. A quantitative yield of cysteine was obtained according to the equation given. The

presence of cysteine sulfinic acid was deduced from its power to liberate iodine from a solution of potassium iodide in hydrochloric acid which neither cystine nor cysteic acid will do under the established condition.

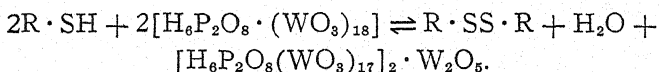
Bloch & Clarke found confirmation of their hypothesis that autocatalytic acceleration of the decomposition of cystine and cysteine in alkaline solution is due to the formation of an intermediate, highly labile compound of the Schiff type formed with pyruvic acid produced during the decomposition. They employed for this study the N-methyl derivatives of cystine and cysteine. According to their hypothesis methylation of the nitrogen should suppress autocatalysis just as acylation had previously been shown to do. They found that decomposition in alkaline solution followed the initial rate without auto-acceleration.

Kassell & Brand (1) have published in detail Kassell's method (1935) for the photometric determination of cystine and cysteine with phosphotungstic acid. This is a modification of Lugg's method (1932) in which the color developed by interfering substances is determined separately in the presence of mercuric chloride which prevents color development by -SH groups. The method is especially valuable since it is applicable to normal urine as well as to hydrolyzates, and since, by the ingenious device of adding cadmium chloride, ascorbic acid may also be determined in a second sample. This procedure was based on the observation that cadmium chloride retards the oxidation of ascorbic acid by mercuric chloride but not by phospho-18-tungstic acid, whereas under similar treatment color development by disulfide and sulfhydryl compounds is completely inhibited.

Schöberl & Ludwig and Kassell & Brand (2) have presented studies of the rates of reaction of sulfhydryl and disulfide compounds with phosphotungstic acid and with sulfite, employing Kassell's photometric method (1935). Both confirm the observations of previous investigators that cysteine and various other thiol compounds produce twice the color with phosphotungstic acid when sulfite is added. The molecular extinction coefficient of reduced phosphotungstic acid is 8200 according to Kassell & Brand but 8100 when calculated from the data of Schöberl & Ludwig. With homocysteine (Kassell & Brand), the reaction was only 75 per cent complete after three hours. Hexocysteine, pentocysteine and  $\gamma$ -thiobutyric acid developed color rapidly during the first two minutes, after which the extinction coefficient, though less than that of cysteine, remained constant. Kassell &

Brand were unable to explain the aberrant behavior of these compounds, but suggested that internal ring-formation might be a contributing factor. As found by Schöberl & Ludwig, rate of color development by disulfides is limited by the rate at which sulfite reduces the disulfide and varies with different substrates. Kassell & Brand utilize this phenomenon in the determination of mixtures of disulfides, particularly the estimation of cystine and homocystine in cystinuric urine. Complete color development (200 per cent) was attained by a series of thiols in the presence of sulfite. With homocysteine the rate was retarded but, in the presence of a large excess of sulfite, reached the same final value, which indicated that the lactone ring is opened under these conditions.

Shinohara offers evidence that the reaction between cysteine and phospho-18-tungstic acid (in excess) may be expressed:



When cysteine is in excess a second equivalent of oxygen is given up by the complex acid with production of greater color. The second reaction takes place slowly in acid solution, rapidly in alkaline.

Bersin & Steudel investigated polarimetrically the thiol-disulfide reaction between *l*-cystine and an optically inactive thiol, thioglycolic acid, and between *l*-cysteine and dithiodiglycolic acid. They found from their polarimetric determinations that the reaction proceeds according to the equation:  $RSSR + 2R'SH \rightleftharpoons R'SSR' + 2RSH$ , coming to equilibrium from both sides at 50 per cent transformation. This reversible oxidation-reduction reaction follows the law of mass action and the free energy change is close to zero. The rates of reaction, which are finite, are determined by the degree of dissociation of the thiols into  $RS^-$  and  $H^+$ .

A further study of the application of Heyrovský's (1932) polarographic method of determining sulfhydryl groups with the dropping mercury cathode has been presented by Rosenthal. Cystine can be detected in as low as  $3 \cdot 10^{-7}$  molar concentration, and since only 0.1 cc. is needed, about 0.077  $\mu$ g. cystine is sufficient. The potential at which cysteine produces its characteristic step in the conductivity curve is  $-1.8$  volts while the sulfhydryl group in proteins produces a similar effect at a somewhat less negative potential. Cystine and cysteine are indistinguishable, as reduction of S-S to -SH occurs at a still lower negative potential. The course of hydrolysis of a protein can be fol-

lowed, the step-up in the conductivity curve gradually shifting from the region characteristic of the protein, to that characteristic of cysteine.

Andrews & Andrews, using the Brand, Harris & Biloon modification (1930) of the Sullivan method for the determination of cystine, found color development inhibited by hydrogen sulfide and a wide variety of organic substances with reducing properties, i.e., adrenalin, tannic acid, ascorbic acid and others, including sugars and aldehydes when present in high concentrations. They suggest conditions for carrying out the Sullivan test with considerably lessened interference from those reductants.

Sullivan & Hess (1) found that aldehydes, present in excess, interfere less with cysteine determinations by the Sullivan method (in 0.1 *N* HCl) than by the Folin-Morensi and the Mason methods, though more than by the Okuda. With long contact (more than four hours), lessened acidity and high concentrations of the reactants, thiazolidinecarboxylic acids which do not react with the Sullivan reagent are formed. Aldehydes do not interfere in the cystine determination (Hess & Sullivan). Pyruvic acid forms a complex with cysteine [Sullivan & Hess (2)] at a certain range of pH, but hydrolysis is quantitatively brought about by boiling for 10 minutes with 2 per cent hydrochloric acid. The same authors (3) and Sullivan, Howard & Hess have suggested modifications of previous methods of hydrolysis of proteins and of finger-nail clippings respectively, by which cystine estimations may be greatly speeded. In the former case hydrolysis in the presence of titanous chloride reduced the humin formation and lessened the time necessary for liberation of cystine and cysteine. With finger-nail clippings, more satisfactory and rapid hydrolysis was accomplished by substitution of sulfuric for hydrochloric acid as the hydrolyzing agent.

Graff, Maculla & Graff have modified the Vickery & White method for determination of cystine, adapting it for microanalysis. Only 2 mg. of cystine are necessary and the procedure may be completed in four hours after hydrolysis of the protein. They substitute hydrochloric for sulfuric acid as the hydrolyzing agent since it is a better solvent for the cuprous oxide used as precipitant, and since addition of extraneous sulfur is avoided. Cystine is estimated either from the sulfur (as barium sulfate) or from the nitrogen content (Kjeldahl method) of the cuprous mercaptide.

Guthrie has improved the method of Guthrie & Wilcoxon (1932)

for the determination of glutathione in tissues. He employed a metaphosphoric acid extract, containing hydrogen cyanide to prevent oxidase action. Hydrogen sulfide was determined iodometrically following oxidation of the glutathione by elemental sulfur according to the reaction:  $2\text{GSH} + \text{S} \rightarrow \text{GSSG} + \text{H}_2\text{S}$ . Chapman has modified Joyet-Lavergne's procedure for the determination of free sulfhydryl and disulfide groups in tissues *in situ*, whereby he has increased the sensitivity and stabilized the color somewhat.

Todrick & Walker (1) proposed the amount of phenolindo-2:6-dichlorophenol reduced by a known weight of protein in an atmosphere of nitrogen, as a measure of free sulfhydryl groups present in the protein. In spite of the difficult end-point, they claimed the method to be accurate to within two or three per cent. Kuhn & Desnuelle utilized similarly the equilibrium: 2 phorphyrexid (red)  $\rightleftharpoons$  porphyrindin (blue) + 2H. They claimed the advantages of clearness of end-point and speed of reaction, the reduction occurring instantaneously in contrast to the 24-hour period required in the method of Todrick & Walker for native proteins. The procedure is carried out in Thunberg tubes at room temperature (see also Greenstein, 4).

Rutenber & Andrews in a study of the applicability of the Benedict-Denis procedure to the determination of methionine sulfur, found that a maximum of 95 per cent of the theoretical yield could be obtained when the combustion was conducted in the neutral range. When solutions with higher or lower pH values were used the percentage recovery fell to as low as 37 per cent.

Kassell & Brand (3) have presented a detailed study of Baernstein's method (1936) for the determination of the sulfur distribution in proteins, introducing modifications in the apparatus as well as in the details of the methods. They have investigated sources of error, such as mercaptan formation and worked out empirical corrections applicable to their technic. In addition, the sulfate sulfur is determined gravimetrically, after which the barium sulfate is reintroduced into the apparatus, digested with hydriodic acid and the sulfur determined as hydrogen sulfide.

*Insulin.*—Space does not permit discussion of the illuminating series of articles by Wrinch (1, 2, 3, 4, 5), Wrinch & Langmuir and Crowfoot on the structure of the insulin molecule as revealed by x-ray analysis.<sup>3</sup>

Stern & White (1) reduced insulin with thioglycolic acid in an at-

<sup>3</sup> Cf. *Ann. Rev. Biochem.*, 8, 119 (1939).

mosphere of nitrogen in acid phthalate-hydrochloric acid buffer of 0.08 *M* strength, at a pH of 1.8 to 1.9 and at 30°. Studies conducted by Svedberg (White & Stern) show that after 150 minutes of treatment no change in molecular weight had occurred. The isoelectric point and the light absorption in the ultraviolet range of the spectrum were essentially unchanged. Since at that time one or two disulfide groups of the hormone were reduced with a loss of pharmacodynamic activity of approximately 50 per cent as determined by the mouse convulsion method, the authors suggested, in accordance with the ideas of various earlier investigators, that one or two dithio linkages in insulin may be specially concerned in its physiological activity, i.e., they represent the activating groups as regulating a functioning center. It seems to the reviewer that the possibility suggested by du Vigneaud must be considered—the architecture of the molecule as a whole is the important factor, and that disruption of any disulfide bond lowers or destroys the potency of the hormone.

Stern & White (2) in acetylating insulin with ketene, found that if the period of interaction is very short (of the order of minutes) only free amino groups are acetylated. The water soluble product formed can be crystallized and retains its hypoglycemic action. When acetylation is continued the hydroxyl groups of the tyrosine gradually become blocked and physiological activity is greatly diminished. The conclusion, that the free amino groups of insulin are not essential for its physiological activity, is at variance with statements of previous investigators (see Hopkins & Wormall, 1934; Jensen & Evans, 1935; and Gaunt & Wormall, 1936) but as pointed out by Stern & White, the previous authors did not determine the integrity of the phenolic hydroxyl groups of their products.

Jacobs, to test whether the hypoglycemic action of insulin was caused by reaction with the sulfhydryl groups of tissues, injected insulin and cysteine separately but simultaneously into fasting rabbits. He found that the hypoglycemic effect of insulin was diminished as would be expected if the administered sulfhydryl groups competed with those of the tissues for the insulin.

Miller & du Vigneaud found that crystalline insulin contains  $3.34 \pm 0.03$  per cent S, equivalent to  $12.5 \pm 0.4$  per cent cystine. The cystine value, determined by the Sullivan method, accounted for all the sulfur.

*Anticoagulants.*—Astrup & Jensen have prepared a highly purified sodium salt of heparin. The activity, *K*, of their product was 7.03;

$[\alpha]^{24}_D = +43.7^\circ$ . Estimated from the elementary analysis of the sodium salt, the composition of their air-dried product, which contained 17 molecules of water, was  $C_{26}H_{78}O_{58}N_2S_4$ . They called attention to the fact that a derivative of mucoitinsulfuric acid with two acetyl groups as previously postulated should have  $C_{28}$  instead of  $C_{26}$ , and suggested that possibly only one acetyl group was present. On hydrolysis, one hexosamine and three sulfuric acid groups were lost, two of the latter apparently being attached to the hexosamine.

Since demonstrating (Jorpes & Bergström, 1936) that the amino sugar of heparin is glucosamine and therefore that heparin is a mucoitin polysulfuric acid, Bergström, Jorpes & Wilander have offered evidence that its activity is to be attributed to the sulfuric acid groups. They found that certain synthetic polysulfonic acids, congo red, germanin, chlorazol fast pink, are also anticoagulants. They state (Jorpes & Bergström) that heparin is not a chemical entity, but a mixture of mucoitin polysulfuric esters with mono-, di-, tri-, and possibly a tetra-ester represented. The activity increases with increasing sulfur content, though a strict proportionality is not evident. They postulate that "the activity of pure heparin must be due to the extraordinarily strong ionic charge in combination with a certain, not too small, molecular size." Chargaff & Olson also ascribe the anticoagulant potency of heparin to its sulfuric acid group since the sodium salts of cellulose disulfuric acid  $(C_6H_8O_{11}S_2Na_2)_x$  and the potassium salts of polyvinyl sulfuric acid  $(C_2H_3O_4SK)_x$  possess anticoagulative action. This is in agreement with the earlier demonstration of Bergström (1936) that introducing sulfuric acid groups into ordinary polysaccharides, cellulose, pectic acid and chitin, by means of chlorosulfonic acid, converts them into active inhibitors of blood clotting.

Chargaff & Olson attempted to prolong the effect of heparin upon injection by combining it with a protamine, salmine, but found all activity abolished. According to Best, Charles & Cowan, the addition product of heparin and salmine is of such a nature that it cannot readily be suspended in water. They utilized a complex of heparin and benzidine and found the anticoagulative effect following subcutaneous administration to anaesthetized dogs much more uniform and prolonged than that of heparin.

Chargaff (1, 2) has described the isolation of an anticoagulant associated with the sphingomyelin fraction of the lipids of sheep and pigs brains. Although the pure compound has not been obtained, Chargaff argues that the lipoidal inhibitor is probably a derivative of

sulfuric acid, since the sulfur content increased at each step of purification, and since cerebroside sulfuric acids of a high degree of potency can be synthesized. He prepared (3) the sulfuric acid esters of cerebrobron and kersasin by the action of chlorosulfonic acid on the cerebrosidates in pyridine solutions and found that they possess marked activity as an anticoagulant. The ester of sphingomyelin was not obtained in pure form because of its relative insolubility, but experiments indicated that it possesses strong anticoagulative power. Meyer & Smyth have isolated from gastric mucin of bulls a mucoitin disulfuric acid which is apparently an isomer of heparin but has only about one per cent of the activity of pure heparin. Ozaki prepared a mucoitin monosulfuric acid from the same source.

In line with these general findings, Wadsworth, Maltaner & Maltaner deny that the inhibitory action of cysteine and reduced glutathione on coagulation is due to their sulfhydryl groups (see Sterner & Medes, 1936) but ascribe it wholly to their titratable acidity.

*Bactericides.*—Investigations of the chemotherapeutic potency in bacterial infections of a wide group of sulfamido and other sulfur-containing benzene derivatives have been vigorously pressed. Since the work of Fourneau *et al.* (1936)), Buttle *et al.* (1936) and Gois-sedet *et al.* (1936) on the relationship of chemical structure to therapeutic activity, few general principles have been established. From the mass of data now being presented much light probably will be thrown on the question of which chemical groups possess toxic properties and which possess bactericidal potency. A limited but well-selected bibliography through 1937 has been presented by Schulte. Fourneau *et al.*, Tréfouël *et al.* and Gray *et al.* have considered especially the relation of structure and activity. Similar publications have been made in 1938 (Buttle *et al.*; Bauer & Rosenthal; Kolloff; Levaditi; Raiziss *et al.*; Crossley *et al.*; Webster & Powers; and McLeod). Crossley and coworkers have given a brief and excellent summary of the present status of the subject. Sulfanilamide, *p*-aminobenzenesulfonamide and prontosil (the disodium salt of 4-sulfonamido-benzene-2-azo-7-acetyl-amino-1-hydroxynaphthalene-3,6-disulfonic acid) have received most detailed attention as to metabolic behavior, toxicity and potency in different types of infections and in various species of hosts.

Methods for the quantitative determination of sulfanilamide have been published by Fuller; Marshall (1); Marshall, Emerson & Cutting (1); Marshall & Litchfield; Proom; Kamlet; Schmidt; Scudi.



Its behavior in the organism has been studied in detail by Marshall and coworkers. According to their findings, sulfanilamide appears quickly in the blood stream following ingestion or subcutaneous injection. It diffuses readily to all tissues and fluids except bone and fat, in which it is present in somewhat lower concentration [Marshall, Emerson & Cutting (2); Engelfried]. In the dog it does not appear to be changed or destroyed to any appreciable extent, whereas in the mouse, rat, rabbit, cat, monkey and man it is acetylated to varying degrees (Marshall, Cutting & Emerson; Fuller). Klein & Harris found that acetylation occurred *in vitro* in the presence of slices of the liver of the rabbit and rat, but not in the presence of muscle, spleen, kidney or blood. The acetylated compound is therapeutically inactive. Sulfanilamide is excreted almost wholly by the kidneys [Marshall, Emerson & Cutting (3); Engelfried; Stewart, Rourke & Allen]. In the dog the sulfanilamide clearance is 20 to 30 per cent of creatinine clearance, determined simultaneously [Marshall, Emerson & Cutting (3)], which indicates a reabsorption of from 70 to 80 per cent of the filtered portion. In man the average clearance in a few determinations was found to be 22.5 cc. per minute. In confirmation of the more indirect evidence of earlier workers (Tréfouël *et al.*, 1935; Colebrook *et al.*, 1936) Fuller showed that sulfanilamide is excreted following administration of prontosil, thus indicating that here also sulfanilamide may be the active principle.

*Neurotoxins of snake venoms.*—The neurotoxins of snake venoms have assumed importance in the chemistry of sulfur compounds since they have been shown to be protein-like substances resembling insulin in respect to their high sulfur content. Separation of the active components of the venom, the neurotoxins, hemolysins, hemorrhagins, and enzymes, has been difficult as many of them are highly unstable. It is also uncertain which properties are represented in the different components.

Slotta *et al.* and Micheel *et al.* have furnished a rich series of papers on these neurotoxins, the former author dealing for the most part with those of *Crotalus* (rattlesnake) and *Bothrops* (genus of pit-viper), and the latter workers mainly with those of *Naja* (cobra) venom. Micheel & Kraft (1935) considered the toxins as prosthetic groups requiring a colloidal carrier. The prosthetic group can be removed by adsorption on various agents or by dialysis, especially after acid hydrolysis. The activity can be restored by addition of protein to the dialyzate. Micheel & Jung (1936) reported that the purified

venom of *Naja flava* (the cape cobra) has a molecular weight of 2500 to 4000, with an isoelectric point of pH 6.5 and an elementary composition of C 45.2, H 7.0, N 14.7, S 5.5 and ash 3 per cent.

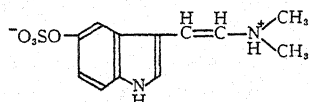
Slotta & Fraenkel-Conrat have succeeded in obtaining a crystalline product, crotoxin, with a molecular weight of about 30,000 (Gralén & Svedberg). According to Slotta & Forster, it contains 13.2 per cent cystine and 1.36 per cent methionine or 36 molecules of cystine and 3 of methionine based on a molecular weight of 33,000. Like insulin, it is inactivated by cysteine in neutral or alkaline solutions. The degree of inactivation corresponds in general to the degree of reduction. Addition of cystine does not restore the activity. Micheel, Dietrich & Bischoff find that the sulfur linkage of cobra venom does not correspond to this type and they postulate that it may be present in a thiolactone or possibly a thiazolidin-like structure. Only about 15 per cent of the activity disappears on treatment with cysteine (Micheel & Schmitz). Slotta, Forster & Fraenkel-Conrat account for all the sulfur of *Naja naja* (3.63 per cent) as disulfide sulfur, but find only about a 25 per cent loss of activity although treated with an enormous excess of cysteine at pH 7.4. They suggest either that the disulfide bridges are extraordinarily stable towards cysteine or that the thiol form is equally toxic.

*Toxins of bee venoms.*—According to Havemann & Wolff (1937) apitoxins (bee toxins) are similar to those of snakes in that they are amphoteric (as demonstrated by cataphoresis), they dialyze through collodion or parchment membranes, and possess similar solubilities. The specimen of apitoxin obtained by Havemann & Wolff had an isoelectric point at pH 8.7. Reinert (1937) has stated that the toxic component resembles a proteose with a nitrogen content of between 13 and 14 per cent and responds positively to various protein tests. It may be inactivated by proteolytic enzymes or by acid hydrolysis. Hahn & Leditschke (1937) separated the toxins into two components, one in the non-dialyzable fraction, a true neurotoxin, and one in the dialyzate, which acts peripherally. In these venoms the toxicity, like that in snake venoms, parallels the sulfur content (Tetsch & Wolff, 1936). The analytical results reported are: C 43.6, H 7.1, N 13.6, S 2.6 per cent.

*Scorpion poisons.*—Tetsch & Wolff (1937) isolated as the hydrochloride 36.5 mg. of a highly toxic amorphous substance from 150 stings of the Syrian scorpion. Here also they found a parallelism between sulfur content and toxicity. In both respects the scorpion poison

isolated by them was intermediate between the toxins of the rattler and of the cobra. The elementary composition was reported as: C 43.6, H 6.8, N 13.6, S 3.8, Cl 5.5 per cent.

*Bufothionine*.—Toad poisons, for the most part, are non-sulfur-containing compounds with an indole nucleus. Bufothionine, however, contains sulfur. It has been found in the secretions of the Japanese toad, ch'an su (Wieland & Vocke, 1930) and *Bufo arenarum* [Wieland, Konz & Mittasch (1934) and Jensen (1935)]. Its chemical constitution has been shown by Wieland & Wieland (1937) to be



Bufothionine

The free base  $C_{12}H_{14}ON_2$ , similar to that obtained on hydrolysis of the sulfur-containing compound, occurs also in some forms of toads but is reported as being physiologically inactive (Jensen & Chen, 1936).

*Enzymes*.—The mass of literature dealing with the rôle of sulfhydryl groups in enzyme chemistry is so extensive that only a few important developments can be mentioned. Hellerman has presented a critical review of the literature up to 1937 covering the evidence that a thiol-disulfide equilibrium is the basis of reversible inactivation of certain hydrolytic enzymes. In this group of enzymes he places urease, papain (see also Balls *et al.*), cerebrosidase, certain hemolysins (see also Cohen *et al.*) and lysozyme of egg white. According to Hopkins & Morgan, lipases are also included. The thiol state is the active one. A number of other hydrolytic enzymes may belong to this group, e.g., ficin, recently crystallized (Walti), which is inactivated by iodine and hydrogen peroxide and reactivated by cysteine. Csonka has offered evidence that the presence of free -SH has an activating effect on peptic and tryptic digestion (see also Voegtlin *et al.*, 1933). Cathepsin (Anson) has been shown not to belong to this group of enzymes, the incorrect conclusion that it is a protease of the papain type being based on experiments with impure preparations.

Gemmill & Hellerman, in a more intensive study of the reversibility of inhibition of glycolysis [previously demonstrated by Lipmann (1933, 1934) and by others] have shown that inhibition of glycolysis in extracts of frog's muscle may be produced with small concentra-

tions of phenylmercuric hydroxide, *p*-chloromercuri-benzoic acid and mercuric chloride and that this inhibition may be abolished by the use of cysteine or reduced glutathione. The block probably occurs at the hexose stage. Inactivation by iodine is also reversible. The reactivators employed were reduced glutathione, cysteine and ascorbic acid. They suggested tentatively that this oxidative-reductive mechanism may function as a control of catalysis of the glycolytic process in frog's muscle. The aldehyde mutase of liver is sensitive to iodoacetic acid (Dixon & Lutwak-Mann). Green, Needham & Dewan, after investigating the triosephosphate, triose and  $\alpha$ -glycerophosphate mutases of rabbit muscle, advanced the hypothesis that each mutase consists of a protein with two active groups, one of which activates the substrate and catalyses the oxidation of the substrate by the coenzyme; the other activates the  $\alpha$ -ketonic acids and catalyses the reduction of the  $\alpha$ -ketonic group by the reduced coenzyme. The oxidative process is suppressed by iodoacetic acid, while the reduction of the ketonic acids is unaffected. This suggests the probability that in the former process the sulfhydryl groups of the enzyme may be involved. Rapkine has added further evidence for the presence of the thiol grouping in the active enzyme which catalyses the oxido-reduction between triosephosphate and pyruvate. Oxidation by iodine or oxidized glutathione greatly diminishes its activity, which may be restored by cysteine or glutathione. The activity of succinic dehydrogenase is completely abolished in a solution of cystine or oxidized glutathione at pH 7.6 and is regained in a similar solution of cysteine or reduced glutathione (Hopkins & Morgan). Its activity is also inhibited by maleic acid which forms addition products with sulfhydryl [Morgan & Friedmann (2)] and by various other compounds which react with sulfhydryl (Hopkins, Morgan & Lutwak-Mann). The same phenomena were not exhibited by  $\alpha$ -glycerophosphate, lactic or malic dehydrogenase, aldehyde mutase or alcohol dehydrogenase of horse liver. These authors did not reinvestigate inhibition by iodoacetic acid.

Runnström *et. al.* report that whereas yeast cells at pH 5 have a respiratory quotient of 1.09 in the presence of glucose or pyruvic acid, upon addition of cysteine the respiratory quotient falls to 0.85 with a change from aerobic metabolism to fermentative metabolism. Baker, on the other hand, finds that glutathione has no effect on the aerobic glycolysis of tumor, brain, testes and embryo, as measured in the Dixon-Keilin manometers and hence finds no indication that glutathione participates in the Pasteur reaction. Morgulis, working with

hog, dog and rabbit bloods, found no strict relationship between the content of reduced glutathione and glycolytic activity. It is possible that physiological concentrations of glutathione can be considerably decreased before glycolytic activity is affected [cf. e.g., Morgan & Friedmann (2) p. 869].

Bersin states that the disulfide state is the active one in certain hydrolytic enzymes, e.g., in the phosphatase from kidney, brain and spleen (see also Thannhauser *et al.*), and in the  $\beta$ -fructosidases,  $\beta$ -glucosidase and amylase. Theorell, in a study of the oxygen carrier, the so-called yellow enzyme, found that denaturation by acid dialysis is associated with appearance of free sulfhydryl groups and that the process may be reversed by subsequent dialysis in distilled water.

The protein in catalase is probably united with the ferric iron of porphyrin through a sulfur linkage (Bersin, 1935; Lyman & Barron).

In spite of the long-standing hypothesis that glutathione plays an extensive part in respiratory mechanisms, its rôle in definite processes is only beginning to be elucidated. Borsook, Davenport *et al.*, in investigating the conditions for reduction of dehydroascorbic acid by glutathione assert that hydrogen may be passed directly from glutathione to dehydroascorbic acid without enzymic action. Cysteine may replace glutathione, although at the same molal concentration it is only one-half as effective. According to these investigators, glucose, dehydrogenase, glutathione, a hemochromagen, ascorbic acid and oxygen constitute a complete respiratory system in which a metabolite is burned and oxygen is used. Schultze *et al.* demonstrate that reduction of dehydroascorbic acid is through thiol groups of both glutathione and fixed compounds. They find no evidence of enzymic reduction in suspensions of liver, muscle, small intestines, blood and erythrocytes of the guinea pig. Crook & Hopkins have brought forth confirmatory evidence of the earlier findings of Hopkins & Morgan (1936) that cucumber and cauliflower juices contain a reducing mechanism which catalyses the reduction of dehydroascorbic acid by glutathione. They suggest that lack of confirmation of the findings of Hopkins & Morgan by Kertesz may be due to differences in the enzyme content of different plants.

Lyman & Barron have shown that oxidation of glutathione may be brought about by naturally occurring hemochromogens and Morrison & Williams have demonstrated the efficiency of glutathione under physiological conditions in reducing methemoglobin to hemoglobin. They suggest that glutathione may be a part of the mechanism by

which accumulation of methemoglobin may be prevented in the intact erythrocyte. (See also Machlis & Blanchard.)

*Metabolic studies.*—Since the announcement by Womack, Kemmerer & Rose that methionine rather than cystine is the sulfur-containing amino acid which is indispensable in nutrition, various other papers bearing on the subject have appeared. A discussion with review of the literature through 1937 is presented by Mitchell.<sup>4</sup> He suggests the explanation "that there is a definite requirement in the growing animal for both cystine and methionine, and that both requirements can be covered by methionine, but only the cystine requirement by cystine itself." That the animal can convert methionine to cystine but not the reverse seems to this reviewer to be more plausible than the suggestion of Womack *et al.*, that "cystine is able to function in place of part of the methionine, but not as a substitute for all." The ability to bring about the necessary transformation probably differs in different animals and may even be lacking in some. Certainly the evidence is strong that at least under certain conditions (i.e., cystinuria), cysteine and methionine have a similar fate which differs from that of cystine. But cysteine and methionine are not equivalent. Brand, for instance, has shown that cysteine cannot replace methionine in the diet of the white rat. In addition to the evidence cited by Mitchell, which is difficult to reconcile with the conclusion that methionine as the indispensable dietary factor cannot be replaced by cystine, may be mentioned an observation by White & Beach. While confirming the conclusion that cystine cannot function for methionine, they noted that on a low methionine, low cystine ration (arachin hydrolyzate) additions of cystine resulted in a slight stimulation of growth. They also made the important observation that homocystine is able to replace methionine and hence is probably converted to methionine. To the reviewer this offers one more bit of evidence that a choice of pathways in metabolism is often available and the path may vary according to the needs of the organism.

Several additional studies have been presented with a view to clarifying the mechanism of the utilization of the sulfur-containing amino acids. Hexocystine and hexomethionine cannot replace cystine for supporting the growth of the albino rat (Jones & du Vigneaud). Homocystine and methionine resemble tryptophane and histidine, in that both *d*- and *l*- forms are available for growth, though with homocystine as with histidine, there is some indication that the *d*-isomer is

<sup>4</sup> *Ann. Rev. Biochem.*, 7, 356-57 (1938).

utilized less readily. The acetyl derivative of *d*-homocystine, unlike that of the naturally occurring *l*-isomer is unavailable (du Vigneaud, Dyer & Jones). The same relationship obtains with the formyl derivatives of *l*- and *d*-methionine, the latter being incapable of supporting growth (Jackson & Block). S-ethylhomocystine (ethionine) cannot be used to replace cystine for growth (Dyer); indeed it even appears to be somewhat toxic.

Methionine, like cystine, but not homocystine, increases the output of taurocholic acid in bile-fistula dogs [Virtue & Doster-Virtue (1)]. Cystamine does not increase the output of taurocholic acid when fed similarly and therefore cannot be involved in the conversion of cystine to taurine (2).

Bennett, working with partially oxidized derivatives of cystine, demonstrated that *l*-cystine disulfoxide is capable of replacing cystine in an animal on a cystine-deficient diet and probably reacts under these conditions  $\cdot\text{SO}\cdot\text{SO}\cdot\rightarrow\cdot\text{S}\cdot\text{S}\cdot+\text{O}_2$  rather than undergoing dismutation. *l*-Cysteine sulfinic acid is unavailable for growth.

The antagonistic actions of cystine in producing an increase in the glyceride storage of livers of rats receiving low protein diets of high fat content, and of methionine in reducing the glyceride storage has been further studied by Channon *et al.* and Tucker & Eckstein (1, 2). Both groups of investigators agree that "the lipotropic action of proteins is the algebraic sum of factors tending to increase or decrease liver fat" as suggested by Channon *et al.* Tucker & Eckstein arrange a series in respect to decreasing lipotropic effectiveness: casein, ovalbumin, fibrin, gliadin and gelatine. The effectiveness is roughly proportional to their methionine content.

In view of these findings, added interest is attached to the recent important announcement by Butts, Blunden & Dunn that *l*-cystine does not contribute to glycogen formation in the liver of the normal rat. It is to be recalled that Vars (1933-34) showed that methionine contributes to extra glucose in the phlorizinized dog. But since from similar studies cysteine also has been reported as belonging to the sugar-producing group (Dakin, 1913), the results in phlorhizin diabetes must be transferred with caution to normal animals and experiments with methionine should be carried out under conditions similar to those set up by Butts *et al.* It also may be significant that lysine, which apparently has no effect on glycogen production, also showed no lyotropic activity. Daggs & Lidfeldt associate the lactogenic properties of cystine with its lyotropic action, but since methionine has a

similar lactogenic effect, these various activities cannot be so simply related.

Since so many metabolic studies are conducted on animals fed by mouth, the observations of Andrews on the production of hydrogen sulfide from sulfur-containing compounds by the intestinal micro-organisms of the dog are of interest.

By determination of cystine with the Sullivan method in ultra filtrates of plasma, using the Pulfrich photometer, Brown & Lewis found that normal human blood contains approximately 1.0 mg. per cent of cystine, a value reduced to from 0.71 to 0.93 mg. per cent after fasting, and increased from 0.80 to 1.05 mg. per cent after meals. In a cystinuric patient 1.13 mg. per cent was observed. The cystine content of rabbit plasma was increased after both cystine and methionine administration (Lewis & Brown).

Dietary methionine behaves as does cystine in increasing the sulfur content of hair (Heard & Lewis; Dawbarn). Because of recent reports that the cystine content of finger-nails is increased following injection of colloidal sulfur, Virtue & Beard tested the ability of sulfur in this form to replace cystine in a cystine-deficient diet and found it ineffective.

Foster, Rittenberg & Schoenheimer found that a small amount of carbon-bound deuterium was introduced into the cystine molecule in albino rats injected with deuterium oxide. The stability of this deuterium in the molecule was not determined. A study of the comparative behavior of cystine and methionine might produce confirmatory evidence of the ability of the organism to amidize those  $\alpha$ -ketonic acids in which both *d*- and *l*-forms are available for growth.

Growth of white rats may be restricted (White) by incorporating iodoacetic acid in the basal diet. Addition of either *l*-cystine or *dl*-methionine counteracts the effect and results in immediate growth. White has announced a program of investigating the mechanism involved. Stannard (1, 2) has found evidence from a study of the comparative effects of iodoacetate and iodoacetamide on respiration and glycolysis and upon other physiological processes in frog muscle, that the mechanism of inhibition by iodoacetyl groups cannot be wholly explained by assuming a combination with an intracellular sulfhydryl compound. Ecker & Pillemer have concluded that the action of iodoacetate in inactivating guinea-pig complement is not wholly upon the thiol grouping of the protein molecule.

Hammett & Reynolds demonstrated the reduction of sulfate to



disulfide in the meristematic tissue of the root tip of *Phaseolus vulgaris*. Schroeder & Woodward showed hydrolysis of both oxidized glutathione and reduced glutathione into their constituent amino acids by enzymic action of rat kidney extract.

*Cystinuria*.—Brand & coworkers demonstrated that cystine, cysteine and methionine, when fed as a constituent of protein (casein, crystalline egg albumin, lactalbumin and reduced lactalbumin) behave both quantitatively and qualitatively as they do when fed in the free form; i.e., cystine is completely oxidized to inorganic sulfate while methionine and cysteine cause a rise in urinary cystine. Kennedy, Lewin & Lunn found, however, that some cystine was excreted as such by a cystinuric patient when 6.4 gm. were fed at a single dose, whereas when the same amount was divided into three doses the cystine elimination was not increased.

Following administration of 8 gm. of *dl*- $\alpha$ -hydroxy- $\gamma$ -methiobutyric acid (during 3 days) to a cystinuric subject, Brand, Block & Cahill (1) recovered 62 per cent of the extra sulfur in the urine, of which 25 per cent was in the form of inorganic sulfate, 29 per cent as cystine sulfur and 43 per cent as unidentified neutral sulfur. An interesting comparison of these results with those obtained from a normal subject may be made. From the urine of a normal subject to whom was administered 3.23 g. of the same compound, there was recovered 94 per cent of the extra sulfur, of which 78 per cent appeared as inorganic sulfate, 2 per cent as cystine sulfur and 14 per cent as unidentified neutral sulfur (Medes). S-methylcysteine (Brand, Block & Cahill, 2) is also oxidized somewhat in cystinuria, though less than by a normal subject. Neither it nor  $\gamma$ -thiobutyric acid nor  $\gamma$ - $\gamma'$ -dithiodibutyric acid yields extra cystine in cystinuria.

*Detoxication*.—In a series of articles by Stekol (1, 2, 3) it has been reported that cysteine, *l*-cystine and *dl*-methionine augment the synthesis of *p*-bromophenylmercapturic acid following ingestion of bromobenzene in dogs maintained on low sulfur diets. Retention of sulfur tends to follow the more immediate output of mercapturic acid. This indicates that tissue protein supplies the cysteine moiety while the ingested sulfur contributes to its replacement. For dogs on an adequate diet, the amount of stores of tissue protein immediately available for this synthesis is a function of body weight (5). Depletion of the stores under continued ingestion of bromobenzene during maintenance on a low-protein diet leads to decreased ability to utilize the mercapturic acid route of detoxication and a rise in excretion of

ethereal sulfate occurs (3). However, fasting rabbits, as has been earlier shown for dogs (Stekol, 1935) and cats (Virtue, 1936) are able to synthesize *p*-bromophenylmercapturic acid from bromobenzene even after a fast of 32 days (Conway). Synthesis of *l*- $\alpha$ -naphthalene-mercapturic acid from ingested naphthalene, and of *p*-bromophenylmercapturic acid from ingested bromobenzene follows the same laws in the rat (Stekol, 4, 6). Glutathione was found not to augment the synthesis of mercapturic acid from ingested bromobenzene in the albino rat, indicating that under the conditions of these experiments, glutathione is not utilized to replenish depleted tissue protein (6).

A newly reported vehicle of detoxication, benzylmercapturic acid (N-acetyl-S-benzylcysteine), following ingestion of either benzyl chloride (dog) or S-benzylcysteine (dog, rabbit and rat) leads to speculation as to the frequency of detoxication by conjugation of cysteine with other aromatic compounds containing loosely bound groups in the side-chains (7).

Williams investigated the detoxication in rabbits of various substituted phenols. When phenol itself was administered in doses from 100 to 250 mg. per kg. the percentage of phenol conjugated with sulfate was constant at about 20 per cent. For doses lower than 100 mg. per kg. the percentage conjugated rose. The degree of conjugation of substituted phenols varied with the position and nature of the substituted groups, acidic ones tending to prevent conjugation while basic ones tended to increase it; in general the effects were found to be strongest with the *ortho*-substituted compounds and weakest with the *para*-.

The author is aware of many omissions in this report necessitated by limitation of space; to mention only a few: inorganic sulfur metabolism (i.e., Borsook, Keighley *et al.*, with radioactive sulfur), pathological metabolism (i.e., Osterberg *et al.*), certain phases of growth stimulation, and a rich source of material dealing with the pure chemistry of many types of compounds of biological interest.

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THE LANKENAU HOSPITAL RESEARCH INSTITUTE  
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## CARBOHYDRATE METABOLISM\*

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### ABSORPTION

Several years ago Verzár and his associates put forth the view that the selective absorption of glucose and galactose was due to their phosphorylation within the intestinal mucosa and as evidence in its support they claimed to have shown that monoiodoacetic acid reduced the absorption rate of glucose to that of xylose, *i.e.*, to a diffusion rate (130). Klinghoffer (78) now reports that the administration of monoiodoacetic acid, even in small doses, produces widespread tissue damage, the most important, as regards absorption, being hemorrhagic enteritis and pyloric spasm. Earlier findings in which it was claimed that the absorption rate of xylose is not affected by the drug were not confirmed; indeed the absorption rates of sodium chloride as well as of xylose were markedly reduced. Although the intestinal damage observed may be sufficient to account for the alteration in absorption produced by the drug, it by no means rules out phosphorylation as an explanation of the selective absorption of carbohydrates, for still other evidence has been marshaled in its support.

A direct relation between thyroid activity and rate of absorption has been clearly established by Althausen & Stockholm (2). Glucose, galactose, and xylose are more rapidly absorbed in hyperthyroidism; thyroidectomy reduces the absorption rate of glucose. The increased absorption rate of the pentose is ascribed to a more rapid emptying-time of the stomach, while in the case of the hexoses these workers believe that hyperthyroidism stimulates phosphorylation in the intestinal wall. Further evidence for the accelerating effect of thyroxine upon absorption is provided by Russell (114), who finds that the rate of glucose absorption in the hypophysectomized rat can be restored to normal with small doses of the hormone. FitzGerald, Laszt & Verzár point out that the selective action of absorption is affected by hypophysectomy. According to these workers the administration of adrenocortical hormone increases the absorption rate of glucose in the hypophysectomized rat (47).

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The absorption rates of a variety of substances have been recorded. The observation that *d*-mannose is absorbed much more slowly than glucose has been confirmed (38). Sorbitol, mannitol and dulcitol are also more slowly absorbed than glucose (19).

Several substances have been shown to influence carbohydrate absorption. Atropine and opium lower the rate (83). Kokas & Ludány (80) find that the absorption of glucose is affected by villi activity, which they believe is under the control of "villikinine."

### BLOOD SUGAR

*Glucose tolerance.*—As early as 1934 Soskin proposed a homeostatic mechanism for the liver "whereby this organ decreases its supply of sugar to the blood in response to an influx of exogenous sugar," and he has since pointed to this mechanism as the principal factor determining the character of the glucose-tolerance curve. Direct proof that such a mechanism exists has now been provided by the experiments of Soskin *et al.* (125), who determined quantitatively the amount of sugar entering and leaving the liver per unit of time, by the aid of thermistromuhr units attached to the incoming and outgoing blood vessels of this organ in specially prepared dogs. The introduction of exogenous sugar, either in a single dose or continuously, produced a condition in which more sugar was found in the blood entering than in that leaving the liver. Somogyi's recent observations (123) on the glucose-tolerance curve are also interesting in connection with this concept, for he has shown that a slight hyperglycemia is a common aftermath of a precipitous fall in blood sugar or hypoglycemia. He suggests that hyperglycemia is a general physiological response to hypoglycemia no matter what the cause and that it reflects an enzymatic balance in the system, glycogen  $\rightleftharpoons$  blood sugar in the liver. These findings are of some importance in the insulin treatment of diabetes if irregularities in blood-sugar concentration are to be avoided, for in this condition the liver mechanism by which the stability of the blood sugar content is maintained is less efficient than in the normal, the hyperglycemic swings in response to hypoglycemia being exaggerated. It may be noted here, in passing, that Ricketts (109) presents evidence which he interprets as supporting the extra-secretion-of-insulin explanation of the glucose-tolerance curve.

Several miscellaneous observations dealing with glucose tolerance are to be recorded. The temporary deterioration in carbohydrate tol-

erance that follows prolonged insulin treatment in non-diabetic subjects has been confirmed (92). Cole & Harned (31) have shown that 70 per cent of adult male rats of the Yale strain have a lower glucose tolerance than rats of the Wistar strain. This abnormality in the former did not appear before fifty days of age. The oral and intravenous methods for studying glucose tolerance have been compared by Koehler & Hill (79). Pijoan & Gibson (103) found that 87.5 per cent of glucose, injected intravenously in a 25-gram dose, is removed from the circulating plasma within four minutes.

*Hepatectomy.*—The fall in blood sugar that follows excision of the liver has been examined in the rabbit by Svedberg, Maddock & Drury (129), and in the monkey by Maddock & Svedberg (91). No striking differences from the extensive observations already made on dogs were noted, although in the case of the monkey the blood-sugar fall was more rapid than that ordinarily observed in the dog. Young *et al.* (138) found that dihydroxyacetone phosphoric acid and hexose diphosphoric acid, as well as a mixture of  $\alpha$ -glycerophosphoric acid and pyruvic acid, were of no value in relieving hypoglycemic symptoms in the hepatectomized dog.

An interesting relation between kidney function and the maintenance of the blood-sugar level in the liverless preparation was discovered by Bergman & Drury (11). They found that excision of the kidneys or interference with their functions markedly increased the glucose requirements of the eviscerated or hepatectomized rabbit. They point out that measurement of sugar utilization in the liverless or eviscerated animal is of doubtful significance unless accompanied by normal kidney function, a fact not recognized in the older observations, particularly those dealing with sugar requirements of the depancreatized, hepatectomized animal.

*Anesthetics and narcotics.*—The difficulty of obtaining a suitable anesthetic for use in the study of carbohydrate metabolism is again pointed out by Hrubetz & Blackberg (71), who investigated the effects of five different barbiturates upon the blood-sugar content. Since these anesthetics led to a decreased epinephrine hyperglycemia, they believe that the barbiturates depress the glycogenolytic power of the liver. According to Ravdin *et al.* (106), the extent of hyperglycemia produced by chloroform anesthesia bears no relation to the initial glycogen content of the liver.

Bodo, Co Tui & Benaglia (14) have extended their earlier investigations of morphine hyperglycemia and now find that morphine fails

to cause hyperglycemia in animals subjected to complete sympathectomy. They suggest that morphine hyperglycemia is the result of the secretion of sympathin. In confirmation of earlier work, Emerson & Phatak (44) and Wada *et al.* (132) report that the blood-sugar response to morphine is diminished when addiction to the drug occurs. According to Corkill & Ennor (33), the hyperglycemia that follows the intravenous administration of non-narcotic doses of magnesium is dependent upon the adrenal gland, for this effect is inhibited by adrenalectomy.

*Neural regulation.*—Bodo & Benaglia (15) produced hyperglycemia by prolonged stimulation of the cardioaccelerator nerves in cats subjected to inactivation of the adrenal glands and destruction of the sympathetic nerves to the liver; they ascribe this finding to the secretion of sympathin. They also deal with its relation to the hyperglycemia of the emotional state. Noltie (102) has shown that the intense hyperglycemia that results from midbrain decerebration is not sustained if adrenalectomy is performed immediately after decerebration. The earlier finding, that transection of the pons after decerebration causes an additional rise in blood sugar, was confirmed; this effect is now ascribed to the adrenal gland.

#### GLYCOGEN

Forsgren's discovery that there are cyclical variations in the glycogen content of the liver has again been confirmed by Sjögren *et al.* (122) and by Deuel *et al.* (39). The latter accept the view expressed earlier by Higgins *et al.* (65) that the phenomenon is the result of diurnal variations in food intake. However, Sjögren believes that an additional factor is involved, which he regards as endogenous in origin.

The glycogenic activity of various amino acids and sugar alcohols has been recorded. Stöhr (127) and Butts *et al.* (20, 21) report measurable increases in the liver glycogen of rats fed *dl*-phenylalanine, *d*-glutamic acid, arginine, aspartic acid and *l*-cysteine. These workers disagree as to whether *dl*-serine and *l*-cystine are glycogen-formers. No change in muscle glycogen was observed by Stöhr after the feeding of twelve different amino acids. The effects of fasting, phlorhizin, exercise, etc., upon the glycogen content of the liver in rats fed diets rich in carbohydrate or protein have been compared by Mirski *et al.* (96). More glycogen remains in the livers of animals maintained on the higher protein diet when they are subjected to the above treatment.

Mannitol is partially stored as liver glycogen in rats (24), but its anhydride, polygalitol, as well as  $\beta$ -D-mannoheptitol (64) are inert in this respect. Sorbitol forms glycogen when administered intraperitoneally, but fails to do so when given *per os* (133).

The glycogenic function of the isolated perfused liver has been reviewed by Lundsgaard (90) who points out that fructose and lactic acid, but not glucose, when added to the perfused cat's liver, lead to glycogen deposition. Since the deposition of glycogen continued only so long as fructose was present in the blood or so long as the blood showed increased lactic acid formation, Lundsgaard postulates that lactic acid is an intermediate phase in the conversion of hexoses to glycogen. But this can hardly be considered a general or obligatory mechanism, for in another species, the rabbit, Corkill & Ochoa (34) showed that the toxemic liver was unable to convert lactate to glycogen, while its ability to form glycogen from orally administered glucose was still retained.

It was shown by Kaplan & Chaikoff (73) that a massive infiltration of fat need not interfere with the glycogenic function of the liver, although it was recognized, as first pointed out by Rosenfeld, that in toxic conditions as well as in abnormal nutritional states a depletion of liver glycogen may accompany an increase in fat. During the year under review this concept of the lack of an obligatory antagonism between glycogen and fat deposition in the liver has been amply confirmed. Marks & Young (94) found that the injection of an anterior pituitary fraction increased both the fat and the glycogen content in the liver, while Ravdin *et al.* (106) have shown that the redeposition of glycogen after anesthesia is accompanied by a continued accumulation of fat.

Chang (28) has confirmed the great reduction in the glycogen and phosphagen content of the rabbit heart that occurs at the moment of asphyxial arrest. In the isolated dog heart, Bogue, Chang & Gregory (16) observed a loss in glycogen during anoxemia when the sugar and lactate contents of the blood were low. The carbohydrate metabolism of the frog heart has been reviewed by Clark *et al.* (30).

The factors that influence the glycogen content of the pancreas were investigated by Hebb (60). The intravenous administration of glucose led to an increase in pancreatic glycogen, the increase in glycogen deposited being related to the amount of glucose injected. The deposition of glycogen observed under these conditions was not facilitated by the administration of insulin.

The glycogen isolated from dog liver was shown by Hassid & Chaikoff (57) to possess the same chemical structure and to exhibit the same general properties as the glycogens from other sources studied by Bell (7, 8), and Haworth & Isherwood (59). The finding that glycogen may be associated with small amounts of phosphorus and nitrogen has led a number of investigators (104) to regard these elements as existing in organic combination with glycogen and therefore as an integral part of the glycogen molecule. Although Pringsheim & Ginsberg (105) claim to have isolated maltose phosphoric acid by the hydrolysis of glycogen with amylase, the acceptance of this finding is made difficult by the failure of other workers to prepare glycogen containing appreciable amounts of phosphorus. Glycogen, practically free of phosphorus, was prepared by Somogyi (124) and by Hassid & Chaikoff (57) among others. Hassid & Chaikoff (58) report, however, that glycogen does react with phosphorus *in vitro*. A product in which phosphorus was shown to be in organic combination with glycogen was obtained when, in several successive steps, glycogen was treated with phosphorus oxychloride in the presence of calcium carbonate.

#### INSULIN AND PANCREATIC DIABETES<sup>1</sup>

*Hyperinsulinism, hypoglycemia and convulsions.*—The recent development of Sakel's insulin-shock therapy for the treatment of schizophrenia has stimulated a voluminous amount of work on the effects of hypoglycemia on brain metabolism, a field which can be only partly dealt with in a review of this nature. The view held by Himwich and others that the oxidative metabolism of the brain, a tissue that utilizes carbohydrate chiefly, is diminished during hypoglycemia has been amply substantiated. Gellhorn *et al.* (50) have shown that hypoglycemia is capable of augmenting the rise in blood pressure consequent upon a given degree of oxygen deficiency and that this augmentation is abolished by the intravenous administration of glucose and, to a lesser degree, of fructose. The similarity in the effects produced by hypoglycemia and anoxemia upon the central nervous system is therefore stressed by Gellhorn (51). This view is also borne out by the recent observations of Himwich *et al.* (69), who were able to obtain amelioration in schizophrenia by employing acute anoxemia. Anoxemia sensitizes animals to hypoglycemia, for Glickman & Gell-

<sup>1</sup> The chemistry and physiology of insulin have been reviewed by Jensen (72).

horn (52) point out that insulin convulsions are more rapidly precipitated when animals are subjected to oxygen deficiency even though the level of the blood sugar at which convulsions occur is not necessarily altered. The dangers of irreversible cerebral changes resulting from prolonged hypoglycemia have been pointed out by Himwich *et al.* (68) and Baker (3) among others. Cerebral damage has been shown to follow prolonged severe hypoglycemia in both animals and man (68, 3, 61).

The shock treatment for schizophrenia has also led to studies on the sugar content of the cerebrospinal fluid, particularly in relation to the simultaneous blood changes (42, 37, 46). In general, there is a lag in the sugar response of the cerebrospinal fluid as compared to blood, the rate of fall in the sugar content of the former being slower and more prolonged.

*Modification of insulin action by metals and other compounds.*—The experiments of Blatherwick *et al.* (12) show that there is an optimum concentration for maximum hypoglycemic effects when zinc chloride is administered with a given dose of insulin. In addition to the concentration of the metal, other factors which determine the augmenting effects of metals upon the hormone are the ratio of metal to insulin and the solubility state of the metal. Scott & Fisher (119) have continued their investigations of the relation of zinc to the insulin content of the pancreas. They show that a deficient insulin content of the pancreas obtained from the diabetic patient is associated with a low zinc content. They have also shown (120) that a high zinc diet does not alter the total insulin content of the pancreas despite the production of pathological changes in the acinar tissue of the gland. An extensive literature has appeared on the clinical use of protamine zinc insulin, but this cannot be treated here.

*Islands of Langerhans.*—Since direct measurements of the insulin content of the pancreas tissues present many difficulties, the histological changes in the islet tissue have often been employed as an index of its insulin activity. The early work of Homans (1915) and of Allen (1922) demonstrated quite clearly that when a strain in the form of a high carbohydrate intake is placed on a remnant of the pancreas (the major part of the gland having been previously excised) degeneration of the remaining islets occurs. The response of the normal whole gland to excessive carbohydrate intake has now been investigated by Woerner (137), who succeeded in effecting the continuous intravenous administration of glucose in guinea pigs for as long as

28 days. Despite the fact that extensive glycosuria and hyperglycemia were not found, the islet tissue showed definite changes. An early decrease in the number of granules in the beta cells was followed by an increase in islet tissue. An increase in beta cells also occurred, not only by mitoses from pre-existing beta cells but also by direct transformation from acinar cells.

*Absorption of insulin.*—The action of various substances in promoting absorption of insulin from the intestinal tract has been actively investigated. Sealock, Murlin & Driver have continued their studies on phenolic compounds (121). Major (93) finds that tetramethylglycol is the most effective glycol compound in permitting the absorption of insulin from intestinal loops, but the response was not striking despite the use of massive doses of the hormone. An earlier finding, that the application by inunction of a glycol compound containing large amounts of insulin in solution produces some lowering of the blood sugar in rabbits, has been confirmed by Abreu & Emerson (1), but this effect is obtained only when a freshly shaven area of skin is used. Lasch & Schönbrunner (84) have shown that certain organic dyes prevent the destruction of insulin by gastric and intestinal juices and that the administration of insulin *per os*, simultaneously with organic dyes and saponin, may be useful in the treatment of diabetes.

*Insulin-resistant states.*—Previous work has established the fact that the blood sugar and liver glycogen fail to give the normal response to insulin in toxic states. In confirmation of these earlier findings, Beamer & Eadie (6) report that insulin is less effective in lowering the blood-sugar content in animals treated with diphtheria toxin and that this resistance to insulin can be annulled by the injection of ergotoxin, which inhibits the sympathetic mechanism. Epinephrine produces a somewhat diminished rise in blood sugar in the toxic animal. Wien (136) has shown that the deposition of muscle glycogen that occurs in the eviscerated spinal cat receiving glucose and insulin can be prevented by the production of fever by means of a bacterial vaccine.

The effects of fasting may also be mentioned here, since evidence was brought forward early to show that the sensitivity to insulin was diminished by the removal of carbohydrate from the diet. Chandler & Chambers (27) compared the insulin response in the same dogs in the well-fed state and after a fasting period of three weeks; they employed the percentage fall in blood sugar as criterion for insulin effect,

which had previously been shown to be more satisfactory than the absolute drop. When compared on this basis, little difference in insulin response was observed between the well-fed and fasted states. A detailed review of carbohydrate metabolism during undernutrition has been presented by Chambers (26).

*Pancreatectomy.*—Further evidence has been brought forward to show that there is no constancy in the degree of diabetes that follows complete excision of the pancreas. Lukens (88) found that complete removal of the pancreas of the young goat produces a very mild form of diabetes, so mild that the excretion of glucose and ketone bodies in the urine is either slight or absent. The so-called classical values for the D:N ratio were not obtained. According to this worker, the completely depancreatized goat is able to utilize some carbohydrate, for only 25 to 50 per cent of glucose administered intravenously was returned in the urine. In the depancreatized monkey, Chapman & Fulton (29) report that sugar may disappear from the urine in the early days of fasting. Constant D:N ratios were not obtained. The monkey is unique in that after pancreatectomy it may survive for as long as 305 days without insulin. According to Dohan & Lukens (41) the severity of diabetes in cats is diminished by thyroidectomy.

The metabolism of isolated slices of diabetic liver, kidney and brain was compared by Himwich and his coworkers (70, 45, 4). The surviving diabetic liver showed an R.Q. of 0.59, a value which agrees closely with previous figures derived from livers of fat-fed or fasted animals. The meaning of these low quotients is discussed (70). An average R.Q. of 0.67 was obtained for diabetic renal cortex and since an increase in oxygen consumption or rise in R.Q. was not obtained in the presence of glucose, Himwich *et al.* (45) concluded that diabetic kidney does not oxidize glucose. They suggest, however, that substances intermediate in carbohydrate metabolism may be oxidized.

The unique position of glucose in the metabolism of brain is again pointed out by Baker, Fazekas & Himwich (4), for even in the presence of suitable doses of nicotine, which almost completely inhibits the oxidation of lactic acid, the diabetic as well as the normal brain continues to oxidize glucose. That insulin is not necessary for the oxidation of glucose by brain even under these conditions is stressed. The utilization of glucose in preference to lactic acid, as shown by diabetic and normal cerebral tissue, does not hold for normal testis or kidney, in which the oxidations of glucose and lactate are inhibited to the same extent by nicotine.



*Mechanism of insulin action.*—Krebs & Eggleston (82) have succeeded in demonstrating an *in vitro* effect of insulin upon minced muscle. In the presence of a boiled muscle extract and certain readily oxidizable substances, such as citrate or some of its derivatives, they find that insulin retards the fall in respiration of minced muscle that sets in after thirty to sixty minutes. This effect of insulin was not demonstrable at earlier intervals despite the use of large doses. The relation of the retardation in respiration by this artificial system to the action of insulin in the intact animal must remain for the present an open question.

*Glycogen.*—The work dealing with the relation of insulin to the metabolism of glycogen has been largely confirmatory in nature and needs only brief reference here. The action of insulin on the glycogen content of the liver and muscle of the depancreatized and normal dog was reinvestigated by Cristol *et al.* (36, 35), who used an electric cautery method (Hédon & Loubatières, 62). This phase is more extensively dealt with by Loubatières (87), who has also reviewed some of the early work on the storage of glycogen by the completely depancreatized dog. The effects of insulin on the glycogen content of liver and muscle in the rabbit receiving glucose by infusion have been recorded by Bridge (17).

#### ANTERIOR PITUITARY

*Glycogenic activity of anterior lobe fractions.*—At least two separate factors from the pituitary are now recognized as necessary for maintenance of glycogen stores in fasted hypophysectomized rats. The fraction that restores muscle glycogen in this animal (glycostatic) has been clearly shown (113, 9) to be distinct from other known pituitary hormones. This action on muscle glycogen is not through the adrenal, since complete maintenance of muscle glycogen in the hypophysectomized rat was achieved with crude extracts in the absence of both adrenal glands (9). The observation that this factor [administered in doses many times greater than the minimum necessary for maintenance of muscle glycogen] had no effect upon the concentration of liver glycogen or blood sugar of the hypophysectomized rat fasted for twenty-four hours, gave rise to the view that still another anterior-lobe hormone is involved in maintenance of liver glycogen. Bennett (10) was the first to show that the treatment of hypophysectomized rats for some days with adrenocorticotrophic hormone resulted in increasing the glycogen content of the liver to values higher than those

found in untreated normal rats. The blood sugar was also increased by the treatments. It is now reported (85, 115) that the administration of Kendall's crystalline compound obtained from the adrenal cortex produces striking increases in the content of liver glycogen and blood sugar of fasted hypophysectomized rats. Muscle glycogen also responded, but despite the injection of doses sufficient to yield very high levels of liver glycogen, normal values for muscle glycogen were not found consistently (115). The carbohydrate stores of the fasted animal are therefore under the control of at least two pituitary factors: the glycostatic, which acts directly on muscle glycogen, and the adrenocorticotrophic, which acts on liver glycogen through the cortical hormone.

*Diabetogenic action of the anterior lobe.*—Young has reinvestigated the effects of anterior pituitary fractions upon hyperglycemia, glycosuria, ketonuria and polyuria, the induction of which after three or four daily injections he regards as the true criterion for the diabetogenic factor (141). A species difference was observed, the dog being most sensitive in this respect. The irregularity of the response even in this animal is noted by Campbell & Best (22). Normal dogs made glycosuric with pituitary extracts differ from depancreatized dogs in their ability to survive without insulin (142). According to Campbell & Best (23) and Richardson & Young (108) the pancreas obtained from normal dogs subjected to chronic treatments with diabetogenic fractions may show definite pathological changes. A well-advanced hydropic degeneration of the islets as well as a lowered insulin content of the pancreas was observed in a dog after seventeen days of injections (23). The so-called pancreatropic hormone of Anselmino & Hoffman is probably not involved here, for the latter's findings are now denied by Santo (117).

Diabetogenic effects have also been demonstrated in partially depancreatized animals. The work of Bennett, Hopper & Linford, who employed partially depancreatized dogs suffering from no apparent disturbance in carbohydrate metabolism, is cited in the review by Russell (112). Glycosuria and ketonuria were consistently induced in such animals by the injection of small amounts of anterior-lobe preparations. By the use of crude extracts Long, Fry & Thompson (86) were able to precipitate glycosuria in partially depancreatized rats, which otherwise failed to manifest any signs of diabetes.

*Anti-insulin action of anterior pituitary fractions.*—The pituitary substance that inhibits the hypoglycemic action of insulin (glycotropic

factor) was found by Young (139, 140) to be not identical with prolactin nor with the diabetogenic factor nor with the thyrotropic and gonadotropic hormones. The anti-insulin effects of crude anterior-lobe fractions are more easily obtained in normal than in hypophysectomized animals (101, 116). This inhibitory factor, however, is unable to influence the spontaneous fall in blood sugar of the fasted hypophysectomized rat in acute experiments (116). In animals in which the liver was excluded from the circulation, it is reported (66) that the glycotropic factor inhibited the peripheral effect of insulin in accelerating the removal of blood sugar, even though the spontaneous fall in blood sugar was not affected by the extract. According to Russell (116), a striking fall in oxygen consumption follows the administration of insulin in fasted and fed hypophysectomized rats, an effect that can be completely prevented by the injection of an anterior pituitary extract. No evidence was found for the view that an increased oxidation of carbohydrate can account for the hypersensitivity of the hypophysectomized rat to insulin. The earlier finding that this sensitivity is not due to a failure in response to the hypoglycemia was also confirmed.

*Respiratory metabolism and the utilization of carbohydrate.*—The fate of fed carbohydrate in the hypophysectomized rat was determined by Russell (111) and Reiss *et al.* (107) by means of respiratory studies. Higher respiratory quotients were observed in hypophysectomized than in normal rats after the administration of glucose. A greater proportion of absorbed carbohydrate was oxidized by the hypophysectomized rat than by the normal. The injection of anterior-lobe extract depressed the R.Q. of both the hypophysectomized and the normal rat fed carbohydrate and hence the proportion of the absorbed glucose oxidized. This effect of the extract in the normal rat was abolished when injections were continued for twenty days. According to Victor & Andersen (131), hypophysectomy lowers both the respiratory metabolism and the R.Q. of isolated liver and renal tissue.

#### ADRENAL

*Adrenalectomy.*—The profound losses in carbohydrate stores that follow adrenalectomy have again been stressed by Britton *et al.*, who have extended their observations to the sloth, dog and monkey (18). Hypoglycemic convulsions may appear in the latter after adrenalectomy. The reduction in cardiac glycogen is also of interest, for this tissue is less sensitive to stimuli that effectively reduce liver and

muscle glycogen. Bennett (9) has revealed a striking difference between adrenalectomy and hypophysectomy in respect to the time when animals lose their ability to maintain muscle glycogen in the fasted state. After hypophysectomy there occurs an immediate loss in the ability of the rat to maintain muscle glycogen, but it requires several days for this defect to appear after excision of both adrenals. The earlier statements of Britton & Silvette on the effects of adrenocortical hormone have been confirmed. According to Grollman (55) the carbohydrate stores of adrenalectomized rats can be maintained at normal levels with this hormone. Katzin & Long (74) also report that intraperitoneal injections of adrenocortical extracts or the crystalline compounds isolated by Kendall from the adrenal cortex produce marked increases in the liver glycogen content of fasted normal mice and rats.

*Role of the adrenal in pancreatic diabetes.*—The interesting observation has been made (67) that ligation of the lumbo adrenal veins is sufficient to lower the intensity of the diabetes that follows pancreatectomy in dogs and cats. All manifestations of the diabetic state, namely glycosuria, ketonuria and nitrogen excretion, are markedly reduced. Himwich *et al.* (67) point out that this is another instance in which some oxidation of carbohydrate may occur in the complete absence of insulin. The course of diabetes in rats subjected to both partial pancreatectomy and bilateral adrenalectomy has been investigated by Long *et al.* (86) and by Grollman (55). In those rats that developed diabetes after partial pancreatectomy it was shown that the second operation reduced the blood sugar to normal and either reduced the glycosuria or abolished it (55, 86). The ingestion of adrenocortical extract or of crystalline compounds prepared by Kendall from the adrenal cortex restored the glycosuria and hyperglycemia in the doubly operated rats. Interestingly enough, the oral administration of large amounts of Kendall's crystalline material would induce glycosuria in rats that failed to develop diabetes after partial pancreatectomy (86). According to Lukens & Dohan (89), the diabetes of the doubly operated dog can also be increased with cortical extract, but large doses are necessary to do this. The hormone producing these carbohydrate changes is believed to be identical with the factor necessary for maintenance of life and of the normal electrolyte pattern (55, 86).

*Adrenal-insulin relation.*—It was shown by Chaikoff & Larson (25) that the rise in blood uric acid observed in the Dalmatian coach dog after the injection of insulin was not a direct effect of the insulin

but the result of epinephrine secreted in response to hypoglycemia. These findings have been confirmed by Miller & Kuyper (95) in the rabbit. Both insulin and epinephrine increase the excretion of uric acid in the rabbit, but the effect of only the latter is direct. The effects of insulin could be abolished by the prior administration of glucose. Apparently there is a species difference in the response, for these workers were unable to influence the uric acid content of blood or urine of man with either hormone. Employing larger doses of insulin, however, Rosenberg (110) reports a fall in blood uric acid after the injection of insulin even though glucose was fed to prevent the development of severe hypoglycemia.

Swann & Fitzgerald (128) have investigated the relative importance of medulla and cortex in the production of the insulin hypersensitivity that follows adrenalectomy. After excision of both glands, rats became 24 times as sensitive to insulin as were normals. By far the major part of this sensitivity could be abolished by transplantation of cortical tissue into the ovary. Moreover, the original sensitivity could be restored by excision of the transplant.

*Potassium changes in relation to carbohydrate metabolism.*—Several observations have appeared which closely link potassium changes to those of carbohydrates. It has already been noted above that the crystalline cortin-like compounds separated by Kendall from the adrenal cortex control the electrolyte balance and produce striking changes in carbohydrate metabolism. Continued intravenous administration of glucose or levulose was found by Flock *et al.* (49) to decrease the concentration of serum potassium in normal dogs. A fall in serum potassium also occurred in adrenalectomized dogs receiving injections of glucose with or without cortin or sodium chloride (75). Both insulin (76) and epinephrine can also produce a fall in serum potassium (49, 77).

#### LEVULOSE AND GALACTOSE

*Levulose.*—The value of the levulose-tolerance test as a measure of liver function was re-investigated by Herbert & Davison (63) and Stewart *et al.* (126). The levulose content of the blood was employed; this avoided the masking effects of glucose. The diabetic patient shows a normal tolerance for levulose. These workers believe that the state of the liver is an important factor determining the type of blood-levulose curve obtained after the ingestion of this sugar. It has been recognized for some time, however, that liver is not the only tissue

capable of converting levulose to glucose, and during this year it was shown by Goda (53) that isolated kidney tissue can also effect this transformation. Since they found that the intravenous administration of levulose lowered the glucose-tolerance curve in the depancreatized dog as well as in the normal, Fletcher & Waters (48) believe that an extra secretion of insulin is not involved here, the lowered glucose-tolerance curve being due to fructose stimulating glycogenesis in the liver. Since the parenteral administration of sorbitol exerted a similar effect on the glucose-tolerance curve, Waters (133) postulates that this polyhydric alcohol is oxidized to fructose in the liver.

*Galactose.*—In a continuation of their observations dealing with cataract formation in rats maintained on a diet high in galactose, Mitchell & Cook now report that a low protein diet hastens the rate of development of these lenticular opacities (99). Some relation exists between the fat content of the diet and galactose utilization, for Schantz *et al.* (118) found that in rats maintained on mineralized skim milk, fat prevented the loss of galactose in the urine. In the liver of rabbits fed galactose Kosterlitz has isolated an ester which he now believes to be galactose-1-phosphate (81). But Colowick has shown that the enzymes present in extracts of liver and muscle do not act on synthetic galactose-1-phosphate (32).

#### RELATION OF FAT TO CARBOHYDRATE METABOLISM

*Ketosis.*—Considerable discussion has arisen as to whether carbohydrate inhibits ketosis by ketolysis (increased utilization) or by anti-ketogenesis (decreased formation). Deuel *et al.* (40) concluded from excretion studies that carbohydrate acts by way of ketolysis. This view is denied by Mirsky (97, 98, 100), who questions whether the metabolism of ketone bodies in the entire animal can be interpreted from the ketone-body content of the urine. In experiments involving the determination of the acetone-body content of the entire nephrectomized rat, Nelson, Grayman & Mirsky (100) showed that glucose did not significantly increase the rate of utilization of  $\beta$ -hydroxybutyric acid. In confirmation of earlier work, Dye & Chidsey (43) point out that injected glucose does not increase the disappearance of administered sodium acetoacetate from the blood of nephrectomized animals. In the heart-lung preparation, which has the advantage of allowing the glucose and ketone-body contents of the perfusing fluid to be accurately controlled, Waters, Fletcher & Mirsky (134) found that the utilization of acetone bodies proceeded vigorously in the absence of

blood glucose and that the addition of glucose to the blood did not increase the rate of ketone-body utilization. Barnes *et al.* (5) also report a failure to correlate the levels of blood sugar and the rate of utilization of  $\beta$ -hydroxybutyric acid by the heart-lung preparation.

*Conversion of fat to carbohydrate.*—Several years ago Gregg (54), working in Murlin's laboratory, pointed out the physiological limitations involved in perfusion studies of the liver and concluded that "it is well nigh impossible to use such a system to demonstrate the neogenesis of carbohydrate from fatty acids on a quantitative basis." Nevertheless, Blixenkrone-Möller (13) now claims to have demonstrated this conversion by artificially perfusing the isolated cat's liver under special precautions. He reports that 20 per cent of added butyrate was converted to ketone bodies, whereas the rest was transformed to sugar. In view of the highly controversial state of this subject, confirmation of these perfusion studies will be awaited with interest. The claim of Haarmann & Schroeder (56) that several tissues can convert  $\beta$ -hydroxybutyric acid to lactic acid is questioned by Weil-Malherbe (135) who, so far as kidney slices were concerned, could find no evidence for the transformation when precautions were taken to remove interfering substances before lactic acid determinations. The conclusion that gluconeogenesis from fat is still an unsettled question seems justified.

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# LIPID<sup>1</sup> METABOLISM

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## NEUTRAL FAT

### ABSORPTION

The concept that phosphorylation in the intestinal mucosa is an essential part of the mechanism of fat absorption has been a dominant factor in this field since the demonstration by Sinclair (1) of a pronounced change in the composition of the phospholipid fatty acids of the intestinal mucosa during fat absorption. This conclusion, originally based on a study of iodine numbers, was later supported by the finding of elaidic acid in large amounts in the mucosa phospholipids during absorption of the unnatural fatty acid (2)—a result which was confirmed by Kohl (3). To be thoroughly convincing it will be necessary to prove that elaidic acid appears sooner or in higher concentration in the mucosa phospholipids than in those of other tissues. In Kohl's experiments this is not evident; the labelled fatty acid had accumulated in the phospholipids of the liver and carcass in considerable amounts at the earliest time during absorption at which its presence in the intestinal mucosa was recorded.

The phosphorylation theory of fat absorption has been supported vigorously by Verzár in an extensive series of investigations (*cf.* 4) showing that fat absorption was inhibited in rats by adrenalectomy (5), and by poisoning with monoiodoacetic acid or phlorhizin. The effect of all of these procedures was attributed to an interference with phosphorylation. Verzár *et al.* (6) extended the studies on adrenalectomy to cats and dogs and found a decreased absorption of fat in four adrenalectomized cats, all of which succumbed during the experi-

<sup>1</sup> The nomenclature of the fatty substances is still in an unsatisfactory state. The term "lipid" has been accepted by most American biochemists as a general name for all naturally occurring substances of a fatty nature and it is so used in this review. The term "fat," which has been employed in the title of previous reviews on this subject, is also used in the same sense by many investigators; but to the organic chemist "fat" means only triglycerides of fatty acids. (For a more detailed discussion of the nomenclature of fatty substances see *Textbook of Biochemistry*, edited by Harrow, B., and Sherwin, C. P., pp. 109, 110, 116, 122, 129 [W. B. Saunders, New York, 1935].)

ments. In interpreting such results it must be kept in mind that these were dying animals in which profound changes in metabolism were taking place. It was impossible to demonstrate any appreciable effect of adrenalectomy on fat absorption in dogs—a result which the authors explained on the ground that terminal circulatory disturbances developed before the inhibition of fat absorption had time to manifest itself. This is difficult to correlate with Verzá's hypothesis that the fundamental disturbance in the adrenalectomized animal is a general failure of phosphorylation processes (7).

Evidence casting serious doubt on the validity of Verzá's interpretation of his findings has accumulated: (a) Klinghoffer (8) found severe intestinal pathology and a decreased absorption of sodium and xylose in the rat following the administration of moniodoacetic acid in the amounts used by Verzá. (b) Lambrechts (9), in an extended investigation, reached the conclusion that phlorhizin exercises its effects not through a specific inhibition of phosphorylation but through a toxic action on cells. (c) Deuel *et al.* (10) showed that the absorption of glucose by adrenalectomized rats (also stated by Verzá to be inhibited through interference with phosphorylation) is normal if the animals are maintained in good condition by sodium chloride administration.

Kohl (3, 11) found that elaidic acid was absorbed at a constant rate when given to rats as the sole source of food or as 40 per cent of the calories of the diet during periods up to eight days in length. McCay & Paul (12) determined the degree of absorption in guinea pigs of a number of fats fed at the low level of 6 per cent of the diet. In general the absorption decreased with increase in melting point (*e.g.*, 87 per cent for cottonseed oil and 74 per cent for hydrogenated cottonseed oil) in contrast with the result in rats (97 per cent for cottonseed oil and 95 per cent for hydrogenated cottonseed oil).

Vonk *et al.* (13) confirmed the conclusion of Breusch (14), in disagreement with Verzá & Kúthy (15), that fatty acids dissolved in bile salts do not diffuse through parchment. A histological study by Leach (16) produced no evidence that leucocytes take part in fat absorption.

#### TRANSPORT IN BLOOD

Except for the procedure proposed by Bloor *et al.* (17), which depends on a selective saponification of neutral fat by castor-bean lipase, no method has been available for the direct analysis of neutral fat in blood. Hence most published values have been determined

indirectly as more or less incidental observations in the study of other lipids (see p. 244).

Astonishingly high concentrations of lipid were reported by Chaikoff *et al.* (18) in the blood of laying hens, either on low or high fat diets, with a maximum of nearly 5 gm. per 100 cc. The increase was largely in the neutral fat fraction, though the phospholipid concentration was also considerably higher than in male and immature female birds. Administration of gonadotropic hormone (pregnant mare serum) (19) or of estrin (20) to immature females produced the same marked rise in serum lipids as was found during laying. With estrin a rise was noted within twelve hours. Particularly striking was the finding of a similar increase in blood lipids in male birds after estrin.

High fat diets produced little or no increase in serum "fat" (21), as determined in dogs by the modified Babcock procedure of Allen; but a large and unexplained increase followed the feeding of crude phospholipid.

Hansen & Brown (22) reported a tendency in rats for the iodine number of the serum lipids to vary directly and the total amount of serum lipids to vary inversely with the iodine number of the dietary fat and suggested that there is a selective retention of unsaturated fatty acids in the blood.

In dogs poisoned for three months with carbon tetrachloride a normal increase in blood plasma fatty acids followed the administration of linseed oil but there was no rise in iodine number as in normal animals (23). The result was ascribed to an inability of the liver to desaturate fatty acids normally, with the consequent rapid removal of absorbed unsaturated fatty acids to supply the needs of the tissues.

#### EFFECT OF DIETARY FAT ON TISSUE FAT

In investigations to which reference has already been made (3, 11), Kohl fed elaidin at levels of 40 and 100 per cent of the dietary calories to rats for periods up to eight days in length. Elaidic acid appeared in the neutral fat and phospholipids of the liver and of the remaining carcass within eight hours. The finding of appreciable amounts in the fat stores, even though elaidin was the sole source of exogenous energy and the animals were losing weight, may be interpreted, in accord with findings of Schoenheimer & Rittenberg [*cf.* (24) for a summary of their classical studies of lipid metabolism with deuterium as an indicator], as indicating that depot fat is in a state of dynamic equilibrium with the fat undergoing metabolism. It must be

remembered, however, that such experiments are not physiological, firstly because the diet is abnormal, consisting only of fat, and secondly because elaidic acid does not occur in nature. Although from its rapid incorporation into phospholipids it would appear to be metabolized like the natural fatty acids, it is possible that it is burned at a lower rate and that under the same conditions natural fat would have been burned as fast as it was absorbed. The accumulation in the depots can hardly represent a storage between feeding periods since the rate of absorption was shown to be constant throughout these experiments. The possibility that the elaidic acid which disappeared (a large proportion of the amount absorbed did not appear in the tissues) was not burned but underwent isomerism to oleic acid was rendered improbable by the finding that it required thirty days on a normal diet to clear the tissues of elaidic acid deposited in three days (25). From Kohl's data the half-life of elaidic acid in the stores appears to be approximately eight days, a time identical with that reported by Rittenberg & Schoenheimer (134) for deuterium-containing fatty acids.

In an extended series of experiments in which deuterium was used as an indicator by the methods of Schoenheimer and his colleagues (except for a new procedure for the final measurement of deuterium concentration) Best *et al.* (26) obtained evidence which indicates, in contrast with the concept of a rapid turnover, that under certain conditions fat may lie dormant in the depots for long periods when it is not needed for fuel. Mice were fed with labelled fat for two weeks and then subjected to various experimental procedures. Some of the results were as follows: (a) At the end of the preliminary period the deuterium content of liver fat was (with one exception) considerably higher than that of depot fat; (b) during fasting the deuterium content of liver fat dropped to a level almost identical with that of the stores, which decreased only slightly; (c) on a high carbohydrate or high protein diet there was little change in the amount or deuterium content of depot fat while there was a marked drop in the deuterium content of liver fat; (d) on fasting after carbohydrate feeding there was an increase in the deuterium content of liver fat while the amount in the stores dropped with no change in deuterium content. These and other findings of the study are in accord with the view that an appreciable transfer of fat from the stores to the liver occurs only when the energy requirements of the animal are not being satisfied by the diet. The lack of change in the deuterium content of the stored fat and the tendency of liver fat to reach the same deuterium concentration

during the transfer indicate that depot fat is not removed by a selective process.

A lack of selection in the deposition of fat in the stores was also indicated by work of Lovern (27) on a novel experimental animal—the eel. From a study of the fatty acid composition of the depot fat of eels, fed on herring, it was concluded that, in effect, there had been a dilution of the eel fat by herring fat without any selection of fatty acids but with some hydrogenation.

The composition of the lipids of the entire rat was altered only slightly from normal on a diet of milk alone for eleven weeks after weaning (28). No mention of anemia, which might be expected under such conditions, is made.

#### FATTY INFILTRATION OF THE LIVER

*Effect of hormones.*—Best & Ridout (29) found no evidence that a pancreatic extract (“lipocaic”) shown by Dragstedt to be active in depancreatized dogs contained any substance in addition to choline and the factors associated with protein which affects the deposition of liver fat in the rat. The same conclusion was reached by MacKay & Barnes (30). Indirect evidence against the theory of a “lipocaic” secretion by pancreas was obtained by Ralli *et al.* (31) in dogs in which the pancreatic ducts were ligated. A fatty infiltration of the liver indistinguishable from that of depancreatized dogs occurred.

On the other hand Channon *et al.* (32) concluded from extended experiments on over 400 rats that there is a lipotropic factor aside from choline and protein in the pancreas. Although there was a wide variation in the results in control experiments and the decrease in liver fat with increased administration of choline was not uniform,<sup>2</sup> the finding in most of the many experiments of a smaller deposition of fat in the presence of pancreatic extracts (prepared according to Dragstedt) than could be accounted for by their content of choline or protein makes the conclusion convincing. The “lipocaic” factor was equivalent to about 400 mg. of choline per 100 gm. and accounted for about two-thirds of the lipotropic activity of pancreas. The magnitude of fatty infiltration is not fully brought out by calculation as percentage of fat in the liver because as the fat of the liver increases there is a general increase in liver weight. To avoid this difficulty

<sup>2</sup> Welch & Welch (33) described a procedure for assaying the lipotropic activity in mice by means of which satisfactorily uniform values were obtained.



Channon and Best now calculate their findings as grams of liver fat per 100 gm. of rat weight. A calculation of weight of fat per unit weight of dry, lipid-free liver would probably be equally effective.

Anterior pituitary extracts or fasting caused a much greater increase in liver fat in guinea pigs and mice than in rats (34). Choline or "lipocaic" did not influence the deposition of fat in the liver of rats caused by pituitary extracts (35). Choline also had no effect on the fatty infiltration which follows partial hepatectomy, but adrenalectomy prevented it in large part (36). The conclusion that the pituitary exercises its influence on the deposition of fat in the liver through the adrenal was reached by Issekutz & Verzár (37) on the basis of their finding that in rats poisoned with carbon tetrachloride or phosphorus, hypophysectomy prevented fatty infiltration, which however occurred under the same conditions if cortical hormone was administered. The number of animals was too few and there was too much overlapping of values, especially in the cortin experiments, to make the finding entirely convincing.

A pronounced increase in the neutral fat content of the liver, as well as in the blood (see above), occurs at the onset of maturity in the female fowl (38).

*Effect of protein.*—Tucker & Eckstein (39), using gliadin instead of casein as dietary protein, confirmed their previous finding that methionine has a marked lipotropic action. The antagonistic effect of cystine in increasing liver fat, found in previous studies by the same authors and by Channon with casein as dietary protein, could not be demonstrated. Lysine had no effect on the liver-fat content. The lipotropic effect of methionine was confirmed by Channon *et al.* (40), but only on diets which produced very high liver fat in its absence. The effect of varying amounts of different proteins in preventing dietary fatty livers was studied by Channon *et al.* (41). Tucker & Eckstein (39) pointed out that the order of increasing lipotropic action (zein, gelatin, gliadin, fibrin, edestin, beef muscle, egg albumin, casein, and whale muscle) was, in general, the same as the order of increasing methionine content.

*Effect of undernutrition.*—The effect of fasting and undernutrition on the liver-fat content was the subject of a comprehensive investigation by Best & Ridout (42). In most previous studies in this field the findings were complicated to some extent by a loss in body weight. A diet which maintains weight while producing fatty livers was devised and fed to rats at various levels with and without choline. The findings were complex and difficult to interpret. Within certain limits

the deposition of fat in the liver was proportional to the calorific value of the diet. Little or no fatty infiltration occurred in fasting rats.

*Effect of dietary cholesterol.*—Loizides (43) showed that the addition of cholesterol to a fatty liver-producing diet brought about not only a marked increase in the cholesterol but also in the neutral fat content of the liver.

#### THE RELATION OF VITAMINS TO THE METABOLISM OF NEUTRAL FAT

The complex relationship between fat metabolism and members of the vitamin-B complex is the subject of several publications. McHenry & Gavin (44) showed that thiamine, riboflavin, and rice-polish concentrate have associated effects upon the amount of body fat, which in the presence of these substances was increased in rats disproportionately to the food intake. Halliday (45) demonstrated an increase in liver-fat content in vitamin-B<sub>6</sub> deficiency. Chanutin *et al.* (46) found fatty livers in normal rats fed a diet containing 20 per cent of yeast. As the amount of yeast was increased above this level the concentration of liver fat decreased linearly. In partially nephrectomized rats the livers showed no fatty changes on yeast diets. The authors suggested that the findings may be related to the antagonistic effect of thiamine and choline, as assumed by McHenry (47). Birch (48) showed that both unsaturated fatty acids and vitamin B<sub>6</sub> are concerned in the cure of the acrodynia-like dermatitis of rats and suggested that the function of B<sub>6</sub> is related to the utilization of unsaturated fatty acids. In this connection it is desirable to mention the important contribution of Turpeinen (49) who showed that linoleic, linolenic, and arachidonic acids, and linoleyl alcohol are effective while oleic,  $\Delta^{12:13}$  oleic, erucic, ricinoleic, chaulmoogric, and  $\alpha$ -eleostearic acids are ineffective in curing the "fat-deficiency" disease in rats. Arachidonic acid was the most potent substance tested and it was suggested that the need of the body may be primarily for this acid.

#### FAT OXIDATION

Verkade (50) summarized his elegant studies of dicarboxylic acid excretion and  $\omega$ -oxidation and discussed their relation to the mechanism of fat oxidation. Injection (51) of salts of dibasic acids in dogs gave essentially the same result as was obtained on feeding. The absence of excretion of the higher acids was attributed not to faster oxidation, which, according to Verkade's theories, would lead to an excretion of lower homologues as formed by  $\beta$ -oxidation, but to a

greater capacity of the organism to store these acids and metabolize them at a low rate.

Support for the  $\beta$ -oxidation theory of fatty acid oxidation was furnished by Bernhard (131) who repeated the classical experiments of Knoop with cyclohexyl (instead of phenyl) substituted fatty acids. Derivatives of acids with an even number of carbon atoms burned completely in dogs while those with an odd number yielded benzoic acid, which must have resulted from the dehydrogenation of a saturated to an aromatic ring. Mono-*N*-methyl and propyl amides of dibasic acids with eight and ten carbon atoms burned completely (132); this shows, since the free acids are excreted (Verkade), that the catabolism from the free carboxyl group is faster than the rate of hydrolysis of the amide group. Small amounts of the anilide of succinic acid were found in the urine after feeding the semianilides of adipic and sebacic acids (133).

Blixenkrone-Møller (52) concluded from extended studies in which isolated livers from normal and depancreatized cats were perfused with whole blood that the liver constantly supplies glucose and ketone bodies to the blood as sources of energy for other tissues. From oxygen-consumption data it was calculated that the fatty acids are broken down into four-carbon chains, *i.e.*, by multiple alternate oxidation (*cf.* 53). The ketone body formation varied inversely with the glycogen content of the liver. About one-fifth of butyric acid, added to the perfusion blood, was transformed to ketone bodies; the remainder formed sugar probably via succinic ( $\omega$ -oxidation), fumaric, malic, oxaloacetic, and pyruvic acids (54). From a further study (55), involving perfusion of the hind quarters also, it was concluded that ketone bodies are burned in working muscles of normal and depancreatized cats. Fat may serve as a source of energy for muscle work provided that it is first broken down to ketone bodies in the liver. The conclusion is in accord with the findings of other investigators (56).

From similar perfusion experiments in rabbits, Toenniessen & Brinkmann (57) also concluded that ketone bodies formed in the liver are burned in the muscles. Otherwise, an entirely different mechanism of fatty acid catabolism was proposed:  $\beta$ -oxidation in the liver with formation of acetic acid (and one molecule of acetoacetic acid per molecule of fatty acid) which is carried to the muscles and catabolized via formic acid. Space limitations prohibit further discussion of these highly interesting and complicated contributions.

Arguments for (58) and against (59, 60) the ketolytic (versus

antiketogenic) explanation of glucose action were presented. The relation of the pituitary (61) and of the adrenal (62) to ketosis was studied. A lower rate of ketolysis in liver slices was reported in the monkey than in other species (63).

#### MILK FAT

Studies of lipids in the blood supplied to and leaving the mammary gland (64) indicate that neutral fat is the source of milk fat. Smith & Dastur (65) found in cows a decrease of about 90 per cent in the lower fatty acids of milk with an equivalent increase in oleic acid during fasting. The finding was interpreted to indicate that the lower fatty acids represent by-products of the synthesis of oleic acid.

#### CHOLESTEROL<sup>3</sup>

##### DETERMINATION

The finding that considerably higher values are obtained with the Liebermann-Burchard reaction on unsaponified than on saponified cholesterol esters (*cf.* 66, 67, 68) was reconfirmed (69). In the opinion of the reviewer, neglect of this phenomenon may account to a considerable extent for the notoriously large discrepancies among the results of different investigators of cholesterol metabolism. Gardner *et al.* (70) modified their procedure for cholesterol determination and now generally obtain "a more constant relation between the free and ester fractions (of the serum) in normal subjects, as found by Sperry (71)." A modification of the method of Schoenheimer & Sperry (72) with its application to certain tissues was published (73). The method was also applied to the determination of cholesterol in tissues by Sturges & Knudson (74). In their procedure for skin, in which the tissue is dried at 32 to 36°, considerable esterification of free cholesterol may be anticipated (75); the relatively high concentrations of combined cholesterol which they reported in skin may have arisen from this source. Gardner *et al.* (76) confirmed, qualitatively but not quantitatively, Sperry & Schoenheimer's (77) finding of a lower concentration of cholesterol in oxalated than in heparinized plasma. The discrepancy is undoubtedly associated with differences in the concentration of oxalate which, in the latter work, as in routine hospital practice, was high.

<sup>3</sup> At the suggestion of the Editors particular emphasis is placed on cholesterol metabolism in the present review.

## IN BLOOD

*In health.*—The wide variability of the total cholesterol concentration of the serum in healthy human beings was emphasized again by Mühlbock & Kaufmann (78) in determinations in 97 women during the intermenstruum. The values ranged from 147 to 322 mg. per 100 cc. (*cf.* 79, 80). In contrast with the results of Page *et al.* (79) in healthy men a rather large increase in the average concentration appeared to occur with increasing age, but in view of the wide variation it will require many more subjects before the finding may be regarded as established. A marked rise in the total cholesterol of the serum during the first four days of life (*cf.* 81, 82) and during pregnancy, and a drop during menstruation were also reported (78). Offenkrantz (83) found in most subjects a decrease in the serum cholesterol at the beginning of the menstrual flow with a rise to above the previous level just after it. Between periods the values were usually quite constant, a finding which with other evidence led the author to state "that cholesterol levels of blood of healthy individuals are, among other considerations, a product of the constitutional: psychiatric makeup." Similar conclusions were reached by Gildea *et al.* (84) and Sperry (80) but later disputed by Man & Gildea (85).

*In disease.*—In view of the wide variation in the cholesterol concentration of the serum or plasma in health apparent deviations from normal in the presence of disease must be interpreted with caution. Much work is rendered valueless by the lack of adequate controls. For example Yoshizumi (86) determined plasma cholesterol in 73 patients with liver disease and compared the results with "normals" in whom the values ranged from 120 to 155 mg. per 100 cc. (*cf.* 78, 79, 80).

Poindexter & Bruger (87) reported a significant elevation in plasma-cholesterol concentration in arteriosclerotic and hypertensive, but not in rheumatic, patients. The finding is contrary to other evidence (*cf.* 88, 89). Boyd & Connell (90) found that the decrease in the ratio of combined to free cholesterol of the plasma in liver disease is part of a general lipopenia.

The little-studied unsaponifiable fraction of the serum lipids was determined, along with cholesterol, by Graff (91) in 150 patients suffering from a variety of diseases. Wide and random variations from 25 to over 400 mg. per 100 cc. were encountered. The cholesterol values were generally low, with many under 100 mg. per 100 cc.

Ludewig & Chanutin (92) showed a direct influence of renal damage on cholesterol metabolism by their demonstration of an in-

crease in the cholesterol concentration of the plasma in partially nephrectomized rats. The finding may be of considerable importance in the study of nephrosis.

Thyroxin administration had no effect on the plasma-cholesterol concentration of normal dogs but it lowered the elevated values in thyroidectomized dogs to the original level (93). The proportion between the cholesterol fractions was not altered by the experimental procedures. In normal rabbits thyroidectomy had little effect on blood-cholesterol concentration (94) but in rabbits which had been fed cholesterol the operation was followed by a prompt rise, even in animals which had not responded to the feeding ("cholesterol resistant") by an increase in blood cholesterol. Conversely, thyroxin lowered dietary hypercholesterolemia both in animals with and without their thyroids.

#### EXPERIMENTAL ARTERIOSCLEROSIS

The development of experimental arteriosclerosis in rabbits following cholesterol feeding was not prevented by the thyreotropic factor of the pituitary (95) nor by choline (96, 97, 98). However, evidence suggesting that choline causes reabsorption of atheromatous lesions was obtained (99).

#### SYNTHESIS AND HYDROLYSIS OF CHOLESTEROL ESTERS

Gardner *et al.* (70) and Klein (100) confirmed the finding (101) of an esterification of free cholesterol on incubation of blood serum. Klein reported a marked hydrolysis of cholesterol esters of serum at pH 5.3 by saline extracts of various tissues. Above pH 7 there was no hydrolysis and usually some esterification (*cf.* 102). With pancreas extracts the situation was reversed—hydrolysis at pH 7, but not at pH 5.3. (In unpublished experiments the reviewer has also observed hydrolysis of cholesterol esters in serum by pancreatic extracts.) The hydrolysis at pH 5.3 was inhibited by bile salts and sodium oleate. It was concluded that one cholesterol esterase is present in pancreas while a different enzyme occurs in all other tissues studied. Neither of these appears to be identical with the esterifying factor found in human and dog serum or with the hydrolyzing factor present only in dog serum (103).

#### IN TISSUES

The deposition of cholesterol in the livers of cholesterol-fed rats was not influenced by pregnancy (104). The finding of an increased

cholesterol content of muscles in nutritional muscular dystrophy of rabbits was confirmed (105).

#### SYNTHESIS AND DESTRUCTION

The conclusion in the last-mentioned investigation that the increase in muscle cholesterol represents a synthesis cannot be accepted without balance studies in view of the fact that the dystrophy-producing diet contained cholesterol (lard and cod-liver oil).

Eckstein (106) confirmed his finding of a larger synthesis of sterol by rats on a high-fat than on a low-fat diet. In addition he showed that the synthesis was greater with an unsaturated (iodine number, 118) than with a saturated (iodine number, 8) fat. In interpreting the result it must be borne in mind that, although the diets were practically cholesterol-free, all of the rats remained in good health, grew normally, and evidently were able to synthesize all the cholesterol they needed even on the low-fat diet. The reason for and the source of the excess synthesis on high-fat diets remain unexplained.

By demonstrating in well-controlled balance studies that unabsorbable sterols may be recovered quantitatively after passage through the intestinal tract Breusch (107) offered strong, though indirect, evidence that the destruction of cholesterol observed on a high-cholesterol diet does not occur in the gut.

#### PHOSPHOLIPIDS

##### DETERMINATION

Micromethods for the estimation of lecithin, cephalin, ether-insoluble phospholipids, and cerebrosides were devised by Kirk (108), and applied to the determination of these substances in normal human plasma and erythrocytes (109). All of the determinations were characterized by an exceedingly wide variation. A method for cephalin estimation was presented by Macy *et al.* (110). Aside from its considerable theoretical interest the finding of Chargaff (111) that cephalin forms an insoluble compound with salmine over a pH range from 2 to 11 while lecithin does not react below pH 10 offers the possibility of a procedure for their quantitative separation.

##### STUDIES WITH RADIOACTIVE PHOSPHORUS ( $P^{32}$ )

The widespread availability of  $P^{32}$  as an indicator has renewed interest in and changed the whole course of research on phospholipid metabolism. Essentially the same procedure is employed in all labora-

tories. A small amount of inorganic phosphorus containing a known amount of  $P^{32}$  is administered to an animal. After a certain period the amount of the indicator which has accumulated in the phospholipid is measured. The results are expressed either as percentage of the total amount given in the phospholipid of a given tissue or as units of radioactivity per milligram of phosphorus. It has not been possible to calculate, except as a rough approximation, the absolute amounts of newly formed or deposited phospholipid which the indicator represents, because the phosphate ion is not distributed uniformly through the tissues (a large part is taken up rapidly by the bones) and the concentration of the medium in which the synthesis is taking place is not known. (The calculation is possible in studies with deuterium which is uniformly distributed in body water.) The method has been employed, therefore, in studying the relative rates of turnover of phospholipids in different tissues.

Hevesy, who first employed isotopes in biological investigations, summarized recent studies carried out in his laboratory (112). After  $P^{32}$  administration to hens, the highest activity was found in liver phospholipid, next in the blood plasma, and least in the ovary and egg, indicating that phospholipid is formed in the liver and carried by the blood to the ovary (*cf.* 113). From similar experiments it was concluded that the phospholipid of milk originates chiefly in the milk gland, that milk fat does not arise from plasma phospholipid, that the increased phospholipid of the blood during dietary lipemia is not formed in the intestinal mucosa, and, from studies in which  $P^{32}$  was injected into fertilized eggs, that the phospholipid of the embryo is newly formed from inorganic phosphorus. In an attempt to find the source of increased blood phospholipid during lipemia cat livers were perfused with normal and lipemic blood containing  $P^{32}$  (114). More phospholipid was formed with the latter but the amounts were small. No evidence for an appreciable formation of phospholipid in the blood itself was obtained (115).

Artom *et al.* (116) grouped tissues according to the speed at which phospholipid accumulates in them, either by synthesis in the tissue itself or by transport from another tissue where it was synthesized. The results follow: liver and intestine, very rapid accumulation; kidney, spleen, and lung, slower accumulation; heart, muscles, and testicles, slow; central nervous system, very slow. There was considerable variation in the data on which the conclusions were based and some overlapping, especially among tissues of the first two groups, but the tabulation is in general agreement with most findings of other



investigators working with  $P^{32}$  as an indicator. It should be emphasized that such studies allow no conclusions concerning the ability of a given tissue to synthesize phospholipid; the rate of accumulation of labelled phospholipid may be a measure of the rate of transfer from another tissue where it is synthesized, or it may be simply a measure of the rate at which inorganic phosphate diffuses into the cells where the synthesis takes place.

The accumulation of  $P^{32}$  in tumor phospholipid, is reported to be as fast as in liver and kidney (117). A considerably lower rate of accumulation of  $P^{32}$  in the total phospholipid of rachitic than of normal rats was found by Dols *et al.* (118).

The rate of accumulation of  $P^{32}$  in tissue phospholipid was studied in laying and non-laying hens by Chaikoff *et al.* (119). No differences were observed except for a more rapid rate in the blood, oviduct, and ovary of the latter. Chaikoff *et al.* (120) also reported a marked difference between young and adult rats in the rate of  $P^{32}$  accumulation in brain phospholipid. The maximum was reached at about the same time (200 hours) in both, but the level in the young rats was about twice that of the adults. The finding is rather difficult to interpret, however, in view of the fact that the majority of rats in the "adult" series weighed less than 150 gm., with two groups averaging 67 and 106 gm.

#### MISCELLANEOUS STUDIES OF TISSUE AND BLOOD LIPIDS

Lipids were determined by Randall in a number of areas and structures of human brain (121). All of the lipid constituents studied were higher in white than in grey areas. A marked decrease in phospholipid, cholesterol, and cerebrosides and an increase in neutral fat were observed in peripheral nerves in the presence of gangrene (122). Yannet & Darrow (123) reported an increase with growth in the concentration of brain lipids in cats. They related the finding to the deposition of lipids in myelin sheaths.

Macy *et al.* (124) showed that practically all of the lipids of red blood cells are separated with the stroma and that the amount of lipid in a single cell is closely related to the size of the cell. No abnormalities in blood-lipid concentrations were found in asthma and hay fever (17, 125). Boyd determined the range of variation in blood-lipid concentrations in normal rabbits (126) and described a lipemia in rabbits infected with *Streptococcus viridans* (127). A decrease in phospholipid and total fatty acid concentration in the plasma followed

adrenalectomy in cats despite a concomitant loss of water, as evidenced by an increase in protein concentration (128). No effect of androgens on plasma lipids in normal or castrated dogs could be demonstrated (129). As part of a general study of the secretion of milk fat (*cf.* p. 239) Smith (130), using macro methods, noted a marked decrease in the concentration of plasma lipids in lactating cows during fasting; the hypolipemia persisted for several weeks after realimentation. There was no significant change in the amount of red cell lipids.

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# METABOLISM OF PROTEINS AND AMINO ACIDS<sup>1</sup>

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## INVESTIGATIONS INVOLVING THE USE OF ISOTOPES OF HYDROGEN AND NITROGEN

Outstanding progress has been made during the preceding year in the development of procedures for tracing the metabolic routes of amino acids labeled with deuterium or the nitrogen isotope N<sup>15</sup>. With respect to deutero-amino acids, one problem has been to substitute the deuterium for hydrogen in known positions, and in such linkage with carbon atoms that exchange of deuterium and hydrogen does not occur on mere contact with water or during any laboratory treatment of products resulting from physiological experiment. Valine- $\beta$ ,  $\gamma$ -d<sub>2</sub> and leucine- $\beta$ ,  $\gamma$ -d<sub>2</sub> synthesized by Kinney & Adams (50), and homocystine- $\beta$ ,  $\beta'$ ,  $\gamma$ ,  $\gamma'$ -d<sub>4</sub> and methionine- $\beta$ ,  $\gamma$ -d<sub>2</sub> prepared by Patterson & du Vigneaud (51) as well as leucine and alanine substituted with deuterium, probably on the  $\alpha$ -carbon, have been demonstrated by Rittenberg, Keston, Schoenheimer & Foster (52) to exchange little or no deuterium for hydrogen on long boiling with 20 per cent hydrochloric or sulfuric acids. It may be concluded, therefore, that the deuterium as oriented in these compounds is in sufficiently stable union that its replacement by hydrogen in the body may be safely interpreted as due to specific metabolic processes. The stability of the carbon-linked hydrogen of a number of amino acids has also been investigated by exposing the ordinary amino acid to contact with deuterium-containing media under various conditions (53, 52, 54). Stekol & Hamill (53) reported the introduction of appreciable carbon-bound deuterium into tyrosine during its tryptic cleavage from casein in the presence of D<sub>2</sub>O, but Schoenheimer and associates (55) repeated the experiment under more rigorous conditions with entirely negative results. The tyrosine thus derived contained only the 0.02 atom per cent of deuterium present in ordinary tyrosine. A negative result was also secured with the enzyme papain and appropriate substrate. As the latter authors aptly point out, these results with hydrolytic enzymes

<sup>1</sup> The prefixes, *d* and *l*, are employed in this review to denote configuration and not rotation.

have "no bearing on the ability of other types of enzymes (*e.g.*, dehydrogenases) to labilize carbon-bound hydrogen." The knowledge accumulated to date indicates that the use of deuterium will prove increasingly valuable in studies of the intermediary metabolism of amino acids.

Stekol & Hamill (56), after giving drinking water containing two per cent  $D_2O$  to mice for periods up to two weeks, demonstrated that the protein of the adult and growing animals and cystine and tyrosine derived from this protein (adult animals) contained notable amounts of stably bound deuterium. Somewhat similar experiments with rats receiving a casein hydrolysate containing firmly linked deuterium have been reported by Ussing (57). The latter calculates from his analyses that in the course of three days ten per cent of the liver protein and 2.5 per cent of the muscle protein is newly formed. More elaborate experiments of this general type have been carried out by Foster, Rittenberg & Schoenheimer (58). By injection and oral administration of heavy water, the deuterium content of the body fluids of mice was maintained at approximately 1.5 atom per cent throughout experimental periods of 10 to 98 days. The protein material from the animals was hydrolyzed in twenty per cent hydrochloric acid, and ten of the resulting amino acids were isolated, purified and analyzed. Over forty per cent of the carbon-bound hydrogen of the glutamic acid had been replaced by deuterium. Lysine alone in this experiment was found to have taken up none of the isotope. It appears, therefore, that lysine synthesized by the plant is used directly in the elaboration of animal protein. The authors point out that this behavior of lysine is in line with the animal's inability to employ unnatural lysine for growth (23), and that the biological introduction of deuterium in the other amino acids may be due to deamination and reamination or less likely to enzymatic labilization of carbon-bound hydrogen without further alteration of the molecule. Ussing (59) has published an interesting comparison of the amount and rate of exchange between deuterium of  $D_2O$  and protein *in vitro* with that occurring *in vivo* between heavy water in the body fluids and the proteins of various tissues and organs.

Equally significant investigations depending on the use of the nitrogen isotope  $N^{15}$  have been initiated by Schoenheimer and co-workers. This mode of attack was first applied in a study (60) of the metabolic relationship of glycine and hippuric acid, and has now been extended to other problems of intermediary metabolism of amino acids (61). In one experiment, young rats were fed for five days on

a diet containing ammonium citrate enriched with heavy nitrogen, and a limited amount of protein. Glycine, glutamic acid, aspartic acid, proline, histidine, arginine, creatine and lysine were then isolated from the animals' tissues and analyzed. All of these substances except lysine (compare results above with deuterium) showed small increases in heavy nitrogen. Again, the feeding of 14 mg. of nitrogen daily as "isotopic" *dl*-tyrosine, incorporated in a normal casein diet, for ten days to an adult rat led to the excretion of only half the isotopic nitrogen, although the total nitrogen excreted was equivalent to that in the total diet ingested. Excess heavy nitrogen was found present not only in the tyrosine but also in the arginine, histidine and a mixture of glutamic and aspartic acids—all isolated from the body proteins. The lysine secured, as well as the ornithine portion of the arginine, contained only the normal amount of the isotope. The nitrogen transferred from the tyrosine to the histidine was found in the alpha amino group as it must be, of course, in the dicarboxylic acids. These experiments, though not exhaustive in their quantitative implications, are revealing with respect to the utilization of dietary ammonia and the interchange of nitrogen between amino acids. It may be added that the authors also report the preparation of glycine, alanine, norleucine, tyrosine, phenylalanine, glutamic and aspartic acids, lysine and leucine with contents of  $N^{15}$  of two atom per cent as opposed to the occurrence of  $N^{15}$  in natural nitrogen mixture of only 0.37 per cent. The mass spectrometer method employed in these experiments may be applied to 1 mg. of nitrogen and is sensitive to 0.003 per cent  $N^{15}$ .

#### AMINATION AND DEAMINATION

*Enzyme systems.*—A dehydrogenase of animal tissues which catalyzes the conversion of *l*(+)-glutamic acid to  $\alpha$ -ketoglutaric acid is the subject of an extensive contribution by von Euler, Adler, Günther & Das (62) (*cf.* Dewan, 63). The reaction, which is resolved into preliminary formation of the imino acid and then hydrolysis of the latter, is reversible. The first of these steps is invoked by apodehydrogenase (from liver) in the presence of either codehydrogenase I or II. Oxygen, upon addition of methylene blue, may serve as the end acceptor of hydrogen in the deamination, while either alcohol or glucose with its respective dehydrogenase can furnish the hydrogen necessary for synthesis of glutamic acid from ammonia and the  $\alpha$ -keto acid. Tests with a wide variety of amino acids revealed an amazing specificity of the enzyme, which occurs principally in the liver and



kidney, for a single substrate—the natural form of glutamic acid. The authors propose that the metabolic synthesis of amino acids may occur by transfer of amino groups, first fixed in glutamic acid by the process described above, to other  $\alpha$ -keto acids according to the mechanism described by Braunstein & Kritzmann (*cf.* 49). A schematic diagram of this and other possible metabolic relationships is presented. Specific enzymes capable of reversible deamination of *l*(+)-glutamic acid in yeast (64) and in *Esch. coli* (65) and of deamination of this amino acid in germinating seeds (66) have been reported.

A method for treating kidney tissue to yield an enzyme which dehydrogenates *l*(+)-alanine has been devised (67). The coenzyme of *d*(−)-alanine deaminase contains flavin and appears to be a flavin-adenine-dinucleotide (68, 69, 70). An investigation by Rodney & Garner (71) indicates that the *d*-amino acid deaminase, acting in tissue slices of rat kidney and liver, is specific for amino groups in the  $\alpha$ -position. Oxidative deamination of racemic alanine, valine and leucine by slices or fine suspensions of kidney tissue is inhibited at oxygen tensions lower than 60 mm. of mercury (72). Bernheim and coworkers (73) find that the oxidation of *d*-amino acids by washed kidney preparation is promoted by pyrrole and by pyrrole plus methemoglobin. Suitable sources of *l*- and *d*-deaminase have been employed by Closs & Henriksen (74) as reagents in a method for the detection of *l*- and *d*-phenylalanine in biological fluids.

Further contributions to the question of intermolecular transfer of the amino groups ("Umaminierung") have been published by Kritzmann (75), Knoop & Martius (76) and Virtanen & Laine (77). The last investigators have demonstrated the transfer of the amino group from aspartic acid to pyruvic acid with the consequent production of alanine in preparations of crushed pea plants.

*Acetylation in the synthesis of amino acids.*—du Vigneaud & Irish (78) have reported in detail their experiments showing that the levorotatory acetylphenylalanine, excreted by the dog after ingestion of the *dl*-compound, is of the *d* or unnatural configuration, contrary to the conclusion of Knoop & Blanco (*cf.* 47). This finding, that the organism does in fact more readily oxidize the acetyl derivative of the naturally occurring phenylalanine, is most important to the significance of the Knoop theory (47) as to the metabolic synthesis of amino acids through the acetyl derivatives by interaction of the corresponding  $\alpha$ -keto acids with ammonia and pyruvic acid. The authors (78) have further tested the theory by demonstrating that feeding of the

*d*(—)-phenylaminobutyric acid results in the excretion of the acetyl derivative of the *l*(+)-form of the amino acid. The mechanism entails, apparently, the oxidative deamination of the administered amino acid to the ketonic acid which by asymmetric synthesis yields the stated acetyl derivative. This latter experiment also affords proof of the ability of the body to convert an amino acid to its optical antipode.

*Deamination and urea formation in the liver.*—Inasmuch as kidney tissue has been revealed by the tissue-slice method to be extremely active in the deamination of amino acids, Mason (79) has inquired into the extent of this kidney process in the intact organism. Unilaterally nephrectomized dogs with the remaining kidney explanted were used to study the arterial-renal venous difference in amino acid content. No consistent difference was evinced in the animals at various intervals following the ingestion of a high protein diet, and the author concludes that in his experiments the kidney was responsible for less than 10 per cent of the total deamination. Maddock & Svedberg (80) have studied the effect of liver removal in the monkey as reflected in the chemical composition of the blood and urine. Similar experiments were also performed on the rabbit (81). The results, like those secured by Mann and his associates with the dog, again emphasize the quantitative importance of the liver in amino acid deamination and urea synthesis, as well as in the degradation of uric acid. Ikeda (82) has examined the role of citrulline in urea synthesis in the liver by means of the perfusion technique and finds his results in accord with the theory of Krebs & Henseleit.

#### AMINO ACIDS AND DERIVATIVES

*Arginine, histidine and lysine.*—The finding of Arnold, Kline, Elvehjem & Hart (83) that a dietary supplement of arginine accelerated the growth of chicks has been extended by Klose, Stokstad & Almquist (84). Employing a casein hydrolysate from which the histidine, arginine and tryptophane had been removed, they showed that these three amino acids are essential for growth in the chick. The chicks lost weight on the arginine-deficient diet even when supplemented with ornithine, or urea, or both of these, but grew well when arginine was given. Rats, on the other hand, are able to synthesize sufficient arginine to permit suboptimal growth (85). Müller & Bräutigam (86) fed the  $\alpha$ -hydroxy analogue of arginine to phlorrhizinized animals and recovered extra sugar in the urine, thus confirming a previous conclusion of Felix & Müller (*cf.* 48) that this com-

pound, like arginine, could be converted to glucose in the body. Doty & Eaton (87, 88) have found that lysine, arginine and histidine, as contrasted with glycine, are absorbed from the intestine of the rat at rates that are a function of the amount of the amino acid in the gut. Kapeller-Adler & Boxer (89) report that the gonadotropic hormone arrests the histidase activity of the liver, thus apparently affording an explanation for the excretion of histidine by pregnant women.

*Carnosine and octopine.*—The specificity of the  $\beta$ -alanyl radical in the depressor activity of *l*-carnosine was demonstrated by Hunt & du Vigneaud (90) who synthesized *d*-alanyl-*l*-histidine and *l*-alanyl-*l*-histidine and found both peptides inactive. Contrary to statements in the literature, neither *d*- nor *l*-carnosine possesses oxytocic activity toward strips of guinea-pig uterus (91). Since  $\beta$ -*l*-aspartyl-*l*-histidine has been suggested as a precursor of carnosine, du Vigneaud & Hunt (92) made a study of some of its physiological properties. It evinces no depressor action, but like carnosine supports the growth of rats on a histidine-deficient diet. With regard to the physiological origin of  $\beta$ -alanine, it is of interest to recall that legume bacteria contain an enzyme which converts aspartic acid to  $\beta$ -alanine by decarboxylation (93). The diphtheria bacillus, as shown by Mueller (94), can obtain the  $\beta$ -alanine it requires from *l*-carnosine but not from *d*-carnosine. Yudelovich (95) reports that the addition of carnosine to muscle-tissue preparations results in an inhibition of anaërobic glycolysis. Considerable variation in the anserine and carnosine contents of muscles of different species and even of different muscles of the same animal was found in quantitative studies by Zapp & Wilson (96). White muscle contains more of these extractives than red muscle. Carnosine is one of the extractable constituents of the skeletal muscle of the amphibian, *Necturus* (97). Octopine, in addition to agmatine and arginine, has been isolated from the muscles of the octopod, *Eledone moschata*, by Ackermann & Mohr (98). Octopine has no effect on blood pressure, blood-sugar content or the activity of smooth muscle (99). Irvin (100) observed that in autolyzing scallop muscle the octopine content increased simultaneously with a decrease in the amount of arginine.

*Tryptophane.*—In view of previous experiments demonstrating that *dl*-amino-*N*-methyltryptophane may replace dietary tryptophane in the promotion of growth, Gordon (32) has compared the naturally occurring abrine now known to be *l*(+)-amino-*N*-methyltryptophane with the *dl* substance. Abrine was found to be practically equivalent

to tryptophane in producing growth whereas the *d*(—)-component of the racemic methyl derivative exerted little or no effect. These results, together with the fact that both optical isomers of tryptophane are well utilized for growth of the rat, are of possible interest in connection with the specificity and reaction mechanism of the deaminases. Further investigation by Kotake & Ito (101) of *d*-kynurenine has shown that this compound, like its mother substance, *d*-tryptophane, is not converted to kynurenic acid in the rabbit. The finding of Kotake & Ichihara that kynurenic acid is excreted in the bile as well as in the urine has been confirmed in extensive experiments with the dog by Correll, Berg & Cowan (102). However, they point out that when kynurenic acid is itself administered *per os*, failure of preliminary absorption may be important in explaining low recoveries in the urine. Several representative species of the Carnivora have been studied with regard to their ability to produce kynurenic acid (103). In common with the dog and coyote, the fox, hyena, wolf and badger excrete the compound, but none could be detected in the urine of the cheetah, serval and five other species even after the ingestion of liberal amounts of tryptophane.

*Phenylalanine and tyrosine.*—Several new studies have been directed toward the elucidation of the metabolic processes which give rise to the excretion of phenylpyruvic acid by certain mentally defective individuals. Jervis (104) fed a number of amino acids including tyrosine and phenylalanine to a patient with phenylpyruvic oligophrenia. Of these, only phenylalanine caused an increased excretion of phenylpyruvic acid. The *d*-form of this amino acid was somewhat more effective in this respect than the natural *l*-form. Phenyl-lactic acid and acetyl-*l*-phenylalanine also augmented the phenylketonuria. Acetyl-*d*-phenylalanine did not. This latter observation is in accord with the fact that tryptophane and methionine of unnatural configuration, but not their acyl derivatives, may be employed for growth. Jervis concurs with Penrose & Quastel (*cf.* 49) that the disease in question entails a lowered tolerance for phenylpyruvic acid which fails to be oxidized at the rate maintained in the normal metabolism of phenylalanine. As to whether the oxidation involves homogentisic acid as an intermediary stage, it is of some interest that the feeding of this compound to Jervis' patient did not hinder the metabolic disposal of phenylpyruvic acid such as to increase its urinary output. Inasmuch as *d*-phenylalanine stimulates phenylketonuria more than does *l*-phenylalanine, Fölling and coworkers (105) have consid-

ered the hypothesis that *l*-phenylalanine may be converted first to *d*-phenylalanine and thence to phenylpyruvic acid through the action of *d*-deaminase. Their experiments led them to the finding of elevated concentrations of *l*-phenylalanine in both the urine and blood of the patients studied but no *d*-phenylalanine could be detected.

Papageorge & Lewis (106) produced experimental alcaptonuria in rats by continued feeding of *l*-phenylalanine in suitable amount. Observation of this phenomenon has subsequently been reported from two other laboratories (107, 108). The ratio of homogentisic acid to nitrogen excreted by an alcaptonuric child was not found to be altered by stimulation of protein metabolism with thyroid substance until toxicosis became manifest (109). Further evidence that phenylalanine and tyrosine may follow different metabolic pathways has been presented (107). Phenylalanine caused a much more definite deposition of liver glycogen and alone decreased the acetoneuria resulting from the feeding of sodium butyrate. Neither amino acid increased the acetone body excretion.

Rose (9) has established that the unnatural *d*-isomer as well as the  $\alpha$ -keto and  $\alpha$ -hydroxy analogues of phenylalanine can be employed by the rat for growth. Also the acetyl-, butyryl-, and valeryl- but not the trimethyl acetyl- or  $\beta$ -methyl valeryl-derivatives of phenylalanine support growth in lieu of this amino acid (43). Raper has published a brief but excellent review (110) on some problems of tyrosine metabolism.

*Leucine, isoleucine, norleucine and valine.*—Rose (9) has demonstrated that the growth requirements for leucine and isoleucine are satisfied by the administration of the corresponding  $\alpha$ -hydroxy and  $\alpha$ -keto acids. Likewise,  $\alpha$ -hydroxy-isovaleric acid may be substituted for valine. However, the unnatural optical isomers of all three amino acids are incapable of replacing their respective corresponding naturally occurring forms. Lunde & Kringstad (111) could find no basis for the report (112) that a dose of 60 to 90 mg. daily of isoleucine would cure a "pellagra-like" dermatitis in rats. It may be noted that the basal diet employed in these experiments contained 18.5 per cent of casein.

*Threonine.*—The four stereoisomers of  $\alpha$ -amino- $\beta$ -hydroxy butyric acid were synthesized by West & Carter (42). Only one, threonine, with the *l*-configuration of the groups on the  $\alpha$ -carbon, is used by the rat for growth. Mayeda (113) has reported a confirmation of the results of McCoy, Meyer & Rose (7) with regard to the essential

nature of threonine for growth. It is not plain, however, from Mayeda's article as to the manner in which the required amounts of methionine, isoleucine and valine were introduced into the basal diet employed.

*Alanine, serine, cystine.*—Gunther & Rose (114) have published the details of their investigation showing that alanine is not essential in the diet for growth of the rat. A study (115) of the fate of serine and cystine in the fasting normal rat led to the surprising finding that cystine is not converted to liver glycogen and does not exert a ketolytic action. Serine, as would be expected from previous experiments on the phlorhizinized animal, gave rise to a considerable increase in glycogen.

*Glycine and hippuric acid.*—Lintzel & Bertram (116) found in carefully designed experiments that glycine did not spare nitrogen loss from a human subject who ingested a "nitrogen-free" diet of fairly high caloric value. Guinea pigs are claimed to be severely poisoned by pure glycine given orally in doses of 0.2 to 2 gm. (117). Griffith (118) has observed that the administration of either sodium hippurate or sodium benzoate causes a toxic effect in rats, as evidenced by lowering of body temperature. The influence of fasting on hippuric acid synthesis has been studied by Hara (119).

#### REQUIRED NITROGEN: FORMS AVAILABLE AND MODES OF ADMINISTRATION

A recent review by Rose (85) summarizes the results of studies made to date on the nutritional significance of the amino acids. Those necessary for normal growth of the rat are lysine, tryptophane, histidine, phenylalanine, leucine, isoleucine, threonine, methionine, valine and arginine. Elsewhere (9), Rose has presented a tentative report on the minimum amount of each of these required to produce growth at a normal rate. The total comprises 5.8 per cent of the diet. Although it has been demonstrated that the ten amino acids listed above will by themselves support excellent growth, the minimum requirement of the essential amino acids given without other amino acid supplements has not as yet been determined. As Rose points out, such a severe limitation of the dietary intake of all amino acids may bring to light an additional requirement—under these particular experimental conditions—for those amino acids otherwise dispensable from the diet. The amino acids required to maintain nitrogen equilibrium in the adult rat have been found by Corley, Wolf & Nielsen (120) to be the

same, except for arginine, as the ones essential for growth which are listed above. A negative balance was found to follow the dietary omission of any one of the nine acids. That arginine is not necessary for nitrogen equilibrium is in accord with the fact that it is also not essential for slow growth (85). Totter & Berg (121) report that supplements of the natural forms of tryptophane, lysine and histidine under suitable conditions of dietary deficiency, produce growth in the mouse. The response of the mouse to these amino acids and likewise to their optical isomers differs from that of the rat only in degree. The requirement of the chick for arginine, histidine and tryptophane has already been mentioned. Further investigations of the amino acid requirements of different species should prove revealing.

Elman (122) has shown that it is possible to maintain dogs in nitrogen equilibrium over three-day periods through intravenous injection of an acid hydrolysate of casein supplemented with cystine and tryptophane. Sucrose given orally in physiological saline solution furnished 50 to 60 kilocal. per day per kg. of body weight, and a solution containing ten per cent each of the amino acid mixture and glucose was injected hourly during six to ten hours. The author has elsewhere (123) referred to the clinical implication of such experiments. Further work dealing with the utilization of injected plasma protein to maintain body protein has been reported by Daft, Robscheit-Robbins & Whipple (124). A positive nitrogen balance was demonstrated in dogs given a diet supplying only a very limited amount of nitrogen, and injected intravenously once or twice daily with plasma secured from healthy donors of the same species. In one experiment the eighteen-day intake of nitrogen as plasma protein was 31.5 gm. while the corresponding urinary nitrogen output was 23.9 gm. The nitrogen of the diet and feces is stated not to affect the conclusion as to a positive balance. It is suggested that there is a "dynamic equilibrium" between plasma and cell proteins, without necessary metabolism through the amino acid stage. Other experiments (125) showed that injection of plasma protein does not give rise to the excretion of appreciable extra nitrogen or sugar in the urine of phlorhizinized dogs.

The experimental demonstration of the utilization of several amino acids for growth by the rat has stimulated a considerable number of investigations as to what derivatives of amino acids are physiologically available in place of the respective naturally-occurring amino acids themselves. The extensive results of such studies have furnished important data bearing on the processes of intermediary metabolism



and the limitations of the animal's synthetic capacities in appropriating substances closely related structurally to the amino acids. These findings have been gathered together in tabular form (Table I). This summary is designed to include only results secured with derivatives which carry the complete carbon skeleton of the corresponding amino acids, and with derivatives altered at the amino or carboxyl groups. Consequently, many interesting reports with respect to other types of derivatives have been omitted.

The data presented are suggestive of new lines of experiment. For example, the amino acids essential for growth, except lysine, threonine and arginine, are shown to be replaceable by dietary supplements of the corresponding  $\alpha$ -hydroxy or  $\alpha$ -keto acids or both. Animals restricted as far as possible to these derivatives should furnish evidence as to the kind of nitrogen necessary for the construction *in vivo* of the  $\alpha$ -amino group.

#### PLASMA PROTEINS

Whipple and his collaborators (126) emphasize the metabolic importance of reserve materials available in the body for regeneration of plasma protein. They reiterate their belief that plasmapheresis continued over a period of two to six weeks and the feeding of an adequate basal diet are essential to bring about a constant minimal production of plasma protein (*cf.* 127, 128). An interesting finding was that, under these conditions, the addition of gelatin to the basal diet of the dog has little effect on the regeneration process but that gelatin plus tryptophane leads to a marked increase of plasma protein. A different method for testing the potency of foods for serum-albumin regeneration has been described by Weech & Goettsch (129): The serum albumin of dogs is depleted by feeding a low protein diet, to which the material to be tested is later added. By this method beef serum proved to be much more effective than beef muscle or casein for serum-albumin regeneration (130).

Plasma-protein formation may be stimulated also by the intravenous injection of a complete mixture of amino acids, according to Elman (131). Dogs depleted of plasma protein by hemorrhage and fasting showed a response within 24 hours following such treatment. Chanutin *et al.* (132) found little evidence in a series of experiments on rats that the liver is directly involved in the formation of plasma proteins. Laparotomy with handling of the viscera caused changes in plasma-protein concentration similar to those produced by partial



TABLE  
UTILIZATION OF AMINO ACID  
(AS DEMONSTRATED IN EXPERIMENTS WITH THE RAT SUBJECTED

	Amino Acid	Acyl Derivative fo=formyl ac=acetyl pr=propionyl bz=benzoyl car=carbomethoxy		Ester Derivative me=methyl et=ethyl ph=phenyl	Amide Derivative am=amide di-et=di- ethylamide an=anilide	Fatty Acid Ana- logue	Acrylic Acid Ana- logue
	Configuration of						
	<i>l</i>	<i>d</i>	<i>lt</i>	<i>dt</i>	<i>lt</i>	<i>lt</i>	
Histidine .....	+	+					— (2)
Homocystine ..	+	+	ac +	ac —			
Methionine ....	+	+	fo +	fo —			
Phenylalanine .	+	+	<i>dl</i> -forms acetyl + butyryl + valeryl + tri-me-acetyl — (9)				
Tryptophane ..	+	+	ac + pr + bz — car — (17, 10, 18)	ac — (10, 5, 18, 21, 22) (10, 18)	et + ph + (5, 21)	am + di-et + an + (20)	— (25, 6) (19)
Cysteine .....	+	— (12)					
Cystine .....	+	— (11, 34)	ac + fo + bz — (45, 29, 26, 36, 44)		me + (26)		— (3)
Isoleucine .....	+	— (9)					
Leucine .....	+	— (9)					
Lysine .....	+	— (23, 1)					— (1)
Threonine .....	+	— (7, 8)					
Valine .....	+	— (9)					
Glycine .....	(Growth retarded by feeding benzoic acid)						

\* The results given in a few cases are not stated as such or are not emphasized in the papers cited, but appear nevertheless to be a logical interpretation of the combined data available.

† Unless otherwise specified, derivatives referred to were prepared directly from the natural amino acids of *l*-configuration. In some instances, the product, *e.g.* the uramino derivative of tryptophane, may

I  
DERIVATIVES FOR GROWTH\*  
TO A LIMITED SUPPLY OF THE CORRESPONDING AMINO ACID)

$\alpha$ -Ur- amino deriva- tive	Hydan- toin	Betaine	N-Methyl deriva- tive			$\alpha$ -Keto ana- logue	$\alpha$ -Ox- imino ana- logue	$\alpha$ -Hydroxy analogue			
$\alpha$ -Carbon								Rotation			
<i>ll</i>	<i>ll</i>	<i>ll</i>	<i>l</i>	<i>dl</i>	<i>d</i>			<i>Levo</i> - rota- tory	<i>dl</i>	<i>Dextro</i> - rota- tory	
				+		+			+		Histidine
				(37)		(33)			(2, 33)		
				+							Homo- cystine
				(14)							
				+				+	+	+	Methio- nine
				(14)				(40)	(26, 41)	(40)	
						+			+		Phenyl- alanine
						(9)			(9)		
—	—	<i>dl</i> —	+	+	—	+	—	—	+	+	Trypto- phane
(25)	(30)	(25)	(32)	(27)	(32)	(25, 6)	(19)	(24, 38, 19)	(38, 19)	(39)	
									— (30)		Cysteine
	—	—	+		—			—			Cystine
	(36)	(44a)	(46)		(46)			(4)			
						+			+		Isoleucine
						(9)			(9)		
						+			+		Leucine
						(9)			(9)		
				— (31)					— (1)		Lysine
											Threonine
									+		Valine
									(9)		
			sarcosine +					glycollic acid +			Glycine
			(27)					(35)			

have undergone some racemization. This reservation does not apply to acetyl-*l*-homocystine, acetyl-*l*-tryptophane or formyl-*l*-methionine. At all events, results secured with a partially or even a completely racemized product may be considered to be valid for the component of *l*-configuration.

hepatectomy. A decrease in the albumin and increase in the globulin of the plasma following hypophysectomy of dogs is reported by Goldberg (133). These changes, the author believes, are due to the hypothyroidism produced in the absence of the hypophysis.

#### THE NITROGEN METABOLISM OF THE MAMMARY GLAND

The view that the amino acids of the blood are the principal precursor of milk protein apparently needs to be modified in the light of recent experiments pointing toward other metabolic sources of the nitrogen. The observations of Graham and coworkers (134) on the nitrogen partition of the arterial and mammary venous blood of lactating goats speak for blood globulin as the chief source of the nitrogen content of milk. In this connection, it is interesting to note that the active mammary gland itself upon extraction with 10 per cent sodium chloride yields more globulin than albumin whereas the inactive gland gives a preponderance of albumin (135). Shaw & Petersen (136) also conclude that amino acids of the blood can account for only a part of the nitrogen in milk. Their calculations, based on the amounts of calcium and amino acid nitrogen lost from the blood in passing through the secreting mammary gland of the cow, show that about 400 volumes of blood furnish the calcium necessary for one volume of milk, but only one-third of the required nitrogen, if amino acids are assumed to be the only precursor. It is not clear to the reviewers, however, that the calcium and amino acid nitrogen values were compared on a common basis, *i.e.*, either of whole blood or of blood plasma. The production of urea by the active mammary gland (137) may be due to the intervention of arginase which has been found to be present (138).

#### TISSUE PROTEINS

A protein, similar to myosin of striated muscle in exhibiting double refraction of flow, has been isolated from smooth muscle (139). Spencer, Morgulis & Wilder (140) have demonstrated that the collagen content of the muscles of rabbits may be increased nearly three fold as a result of advanced nutritional muscular dystrophy. Addis and associates (141) have found that thyroxine but not dinitrophenol causes an increase in the protein content of the liver, kidney and heart, designated in per cent of total body protein. Block (142) has proposed that keratins of ectodermal origin be classified as ( $\alpha$ ) eukeratins with approximate molecular ratios of 1:4:12 for histidine, lysine

and arginine and (*b*) pseudokeratins which relatively yield less arginine and more histidine and which are less resistant to enzymic digestion. Depending on its color, hair yields different amounts of "leukokeratin," "melanokeratid" and "rhodokeratid." The first and last of these are soluble but the second insoluble in cold dilute alkali (143). Block & Lewis (144) have compared the amino acid contents of cow hair and wool, and have also analyzed chimpanzee hair.

#### MISCELLANEOUS PAPERS

Lack of space forbids more than the citation of several other papers dealing with various aspects of nitrogen metabolism: the formation of urea from glutamine in tissue slices (145); the precursor of urinary ammonia (146); nitrogen metabolism as affected by muscular work (147, 148); endogenous nitrogen metabolism (149, 150, 151); the effect of starvation on nitrogen excretion (152, 153), and on the loss of nitrogen from the body tissues (154).

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# MINERAL METABOLISM CALCIUM, MAGNESIUM, AND PHOSPHORUS

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The present review will be largely an extension of the review on the Occurrence, Transport, and Regulation of Calcium, Magnesium, and Phosphorus in the Animal Organism by Schmidt & Greenberg (1). It is proposed to emphasize the development of new technics, particularly the use of radioactive isotopes as indicators of metabolism and the genesis of new concepts in the field of mineral metabolism.

In the *Annual Review of Biochemistry*, calcium and magnesium metabolism, as such, were last reviewed by Shohl in 1933 (2). Since then, in the course of reviewing other subjects, certain phases of this field have been covered in this publication by Peters, Robbins & Lavietes (3), and by Mitchell (4).

Reviews dealing with the above mineral elements have recently appeared in other publications. Mitchell & McClure (5) and Maynard (6) have reviewed the mineral nutrition of farm animals, Morgan (7) the mineral requirements of women during pregnancy, and Leitch (8) and Farquharson (9) the calcium requirements of man. The problem of bone development has been surveyed by Policard & Roche (10), and the relation of the bone-forming elements to vitamin D has been treated by Shohl (11). Schour (12) and Krasnow (13) have discussed the influence of mineral metabolism on the teeth. Because of space limitations, the present review will omit consideration of certain physiological and pharmacological properties of calcium, magnesium, and phosphorus and of the metabolism of bone and teeth.

## DEVELOPMENTS IN THE STUDY OF MINERAL METABOLISM WITH THE AID OF ISOTOPES AS INDICATORS

The application of isotopic indicators to the study of the metabolism of biologically important substances has led to important results in the field of mineral metabolism. In this case it has not been the naturally occurring isotopes, but the induced radioactive isotopes of the mineral elements that have proved useful. Excellent reviews of

certain phases of this subject have been published by J. H. Lawrence (14, 15), and by Hevesy (16).

Because of the revolutionary nature and potential importance of this subject, the reviewer considers it desirable to depart from his set program to the extent of treating all the mineral elements that have been studied by this technic.

Through the property of emitting radiation, the course of the radioactive isotopes of the commonly occurring mineral elements may be traced in the animal organism from the time they are administered until they are excreted. This offers an important tool for the study of absorption, permeability, storage, distribution, chemical transformation, and paths of excretion of the mineral elements. Another important advantage is that, in general, only very small doses of the substance to be studied need be administered, thus avoiding the criticism that the normal physiological mechanisms are being overtaxed.

In many respects it is more advantageous to use a radioactive than a non-radioactive isotope because the detection of the radioactive isotopes is relatively simple with such devices for measuring intensity of radiation as the electroscope or the Geiger-Müller counter. Also non-radioactive impurities which may be present do not interfere with the measurements and thus very tedious purification processes can be avoided. Chemically, the radioactive isotopes behave in identically the same manner as the natural mixture of isotopes of the elements of the same atomic number because they have the same nuclear charge.

The discovery of induced radioactivity by Joliot & Curie (17, 18), and the development by E. O. Lawrence and his collaborators (19, 20) of the magnetic resonance accelerator, commonly known as the "cyclotron," have made it possible to conveniently prepare suitable radioactive isotopes of most of the mineral elements which occur naturally in the living organism.

The factors which determine whether a radioactive isotope will be suitable for investigative work are its degree of stability as measured by its half-life and the intensity of the radiation it gives off. J. H. Lawrence (15) points out that the duration of life of the radioactivity of the element should be suitably short, so that it may be given in small quantities as a tracer to animals and man without danger. For the ideal isotope, the duration of life should, however, be sufficiently long to enable the fate of the element to be followed until it is eliminated by the organism. Similarly, the intensity of radiation should be sufficiently great to allow the easy detection of the small quantities

of certain of the elements that meet the requirements of the organism. The characteristics of the better known of the radioactive elements which have been produced in the laboratories of the world (15) are given in Table I.

TABLE I  
INDUCED RADIOACTIVE ELEMENTS

Atomic Number	Radioactive Element	Radiation	Half-Life
6	Carbon <sup>11</sup>	Positron	21.3 minutes
7	Nitrogen <sup>13</sup>	Positron	10.5 minutes
8	Oxygen <sup>15</sup>	Positron	2.1 minutes
9	Fluorine <sup>18</sup>	Positron	108 minutes
11	Sodium <sup>22</sup>	Positron	3 years
..	Sodium <sup>24</sup>	Beta and gamma	14.8 hours
12	Magnesium <sup>27</sup>	Beta and gamma	10 minutes
13	Aluminum <sup>29</sup>	Beta	10-11 minutes
14	Silicon <sup>31</sup>	Beta	2.5 hours
15	Phosphorus <sup>32</sup>	Beta	14.8 days
16	Sulfur <sup>35</sup>	Beta	80 days
17	Chlorine <sup>34</sup>	Positron	34 minutes
18	Argon <sup>41</sup>	Beta and gamma	110 minutes
19	Potassium <sup>42</sup>	Beta and gamma	12.8 hours
20	Calcium <sup>45</sup>	Beta	2.4 hours
21	Scandium <sup>46</sup>	Beta	80 days
23	Vanadium <sup>48</sup>	Positron	16 days
25	Manganese <sup>54</sup>	Beta	8 months
26	Iron <sup>59</sup>	Beta	45 days
27	Cobalt <sup>58</sup>	Beta	3 years
29	Copper <sup>64</sup>	Beta	12.8 hours
30	Zinc <sup>65</sup>	Positron	60-100 minutes
31	Gallium <sup>72</sup>	Beta and gamma	23 hours
33	Arsenic <sup>76</sup>	Beta and gamma	26 hours
34	Selenium <sup>81</sup>	Beta	56 minutes
35	Bromine <sup>80</sup>	Beta	4.2 hours
..	Bromine <sup>82</sup>	Beta and gamma	36 hours
42	Molybdenum	. . . . .	36 hours
47	Silver <sup>106</sup>	Beta and gamma	8 days
48	Cadmium <sup>115</sup>	Beta	4.3 hours
49	Indium <sup>114</sup>	Beta	50 days
53	Iodine <sup>128</sup>	Beta	25 minutes
56	Barium <sup>139</sup>	Beta	85.6 minutes
79	Gold <sup>198</sup>	Beta and gamma	2.7 days
82	Lead <sup>209</sup>	Beta	3 hours
83	Radium E or bismuth <sup>210</sup>	Beta	5 days

In the field of mineral metabolism in animals, work has been published in which the radioactive isotopes of sodium, potassium, iodine, sulfur, iron, and phosphorus have been employed.

*The alkali elements.*—The internal administration of radio-sodium in the form of sodium chloride was first performed by Hamilton & Stone (21) on two leukemic subjects. No clinical improvement or toxic effects were observed. Measurement of the concentrations of radio-sodium over a period of eight to sixty hours in the urine, blood, and perspiration was carried out. No radio-sodium was detected in the feces.

Hamilton (22, 23) has studied the rates of absorption of radio-sodium, potassium, chloride, bromide, and iodide ions from the gastrointestinal tract of normal human subjects. The radioactivity of the hand was used to follow the absorption. This was determined by the ingenious use of a small hollow lead box, open at one end to allow insertion of the hand and forearm. The box contained a Geiger-Müller counter tube which counted the number of impulses per second emitted from the hand.

The absorption of the radio-sodium, chloride, bromide, and iodide ions could be detected within three to six minutes after their administration. The activity increased rapidly thereafter, and apparently was complete within three hours. Radio-potassium was absorbed more slowly, six to fifteen minutes being required before it appeared in the hand, and about five hours being required for its complete absorption. Absorption, in the sense that it is used by Hamilton, is represented by the increment in the radioactivity emitted by the hand. Complete absorption was assumed when the emitted radioactivity reached a maximum.

Greenberg, Joseph, Cohn & Tufts (24) found that on administering potassium chloride to fasted adult rats, 90 per cent of the potassium was absorbed from the gastro-intestinal tract within half an hour. The major portion of the absorbed potassium was retained and was incorporated into the muscles and other soft tissues of the body. The incorporated potassium displaced a certain proportion of the potassium previously there, and subsequently appeared to have the same fate as the ordinary potassium of the body. It is of interest to note that the liver takes up a greater fraction of the radio-potassium when it is administered orally than when it is injected intraperitoneally. This may be explained if the predominant path of potassium absorption is the portal venous system.

Glazko & Greenberg (25) have shown that the natural radioactivity of potassium is too minute to have any physiological significance. In the maintenance of the beat of the isolated frog heart, potassium ion could not be replaced by radioactive sodium, phosphate, or even radio  $K^{42}$  ions, if the total amount of potassium was reduced to about 1 per cent of normal. Hamilton & Alles (26) found that the induced radioactive alkali cations did not cause any disturbances, such as change in pulse rate or blood pressure, in the circulation of animals.

*Radio-iodine.*—The physiology of the thyroid gland has been studied by Hertz, Roberts & Evans (27), with the aid of radio-iodine, even though this radioactive isotope has a half-life of only 26 minutes. The results of experiments on a series of 48 rabbits showed a preferential accumulation of iodine in the thyroid gland. Radio-bromine, on the other hand, showed no evidence of preferential accumulation in the thyroid. In goitrous rabbits with hyperplastic thyroids, the iodine retention was increased several fold.

*Sulfur and iron.*—Borsook, Keighley, Yost & McMillan (28) followed the urinary excretion of the labeled sulfur upon administering sodium sulfate to a human subject. Through the use of radio-iron, it has been observed (29) that anemic dogs absorb considerable quantities of orally administered iron, whereas normal dogs absorb almost none.

*Radio-phosphorus.*—Because of its ease of preparation, its relatively long half-life (14.8 days), its fairly high intensity of radiation (making it easy to detect), and the importance of its compounds, phosphorus has been the chief radioactive isotope used so far in the study of metabolism. The greater part of the work with this element has been carried out by Hevesy and his collaborators.

Chievitz & Hevesy (30), by adding radio-phosphorus to food, demonstrated that an appreciable part of the phosphorus found its way into the bones, teeth, muscle, and other organs of both growing and adult animals. The phosphorus taken up by the skeleton during growth became equally distributed in time over the whole of the skeleton, thus demonstrating the dynamic nature of bone formation. When sodium phosphate was injected there was a rapid disappearance of the labeled phosphorus from the circulation. This, according to the authors, was due to adsorption of the phosphate ions by the calcium phosphate of the skeleton—the result of the kinetic exchange of phosphate ions between the plasma phosphate and bone phosphate. The exchange of phosphate ions between blood and bones takes place best

in that part of bone which is most vascular. For this reason a greater rate of deposition was found in epiphyseal than in diaphyseal bone.

Hahn, Hevesy & Lundsgaard (31) have calculated that the average time which a phosphorus atom spends in the body is thirty days.

The indicator technic demonstrates that salts of phosphorus, administered by mouth or parenterally, are excreted both by the intestines and kidney, the proportion varying with the species, dietary conditions, mode of administration, etc. In man it was calculated (30) that about a seventh of the radio-phosphorus of the feces comes from excreted materials entering the intestines through the digestive juices. The total phosphorus excreted into the intestines is probably much more than this. About 12 per cent of the phosphorus, administered with food, appeared in the urine of human subjects within twenty-four hours. After this the rate of excretion was only 2 to 3 per cent per day. An adult rat excreted phosphorus about equally in feces and in urine, 25 per cent being eliminated in the first three days; nineteen days were required to eliminate the next 25 per cent. When radio-phosphorus was injected subcutaneously into a rabbit (31), 45 per cent was excreted in the urine and 11.5 per cent in the feces after a lapse of twenty-seven days. Considerable quantities of phosphorus were found to have been taken up by the placenta and foetus of a pregnant rat (30).

In a closer investigation of the exchange of phosphorus in teeth, Hevesy, Holst & Krogh (32) found that in the rat the labeled phosphate was deposited in close proximity to the pulp from which it was derived, and that it was equally distributed throughout the incisors. In the molars (which do not grow) of adult rats there was a much lower accumulation of labeled phosphorus. Of the labeled phosphorus administered the following percentages were found after three days in one-milligram quantities of tooth ash: Incisor, 0.0044; molar, 0.0018. For a comparison, the content in the tibial bone was 0.0064 per cent. In the cat, three to four times as much labeled phosphorus was present in the canines as in the molars of young animals sacrificed a few hours after its subcutaneous administration. In adult animals, the quantities were about equal in the different teeth several days after the injection. In a sixteen-year-old human subject it was found that only about one in 300,000 of the administered radio-phosphorus atoms enters a single tooth. The replacement of 1 per cent of the phosphorus content of a human tooth by phosphorus atoms from the food is calculated to take about 250 days.

One of the most interesting studies carried out with this technic has been that of Aten & Hevesy (33) on the role of phosphorus in the formation of milk. When radioactive phosphorus, as sodium phosphate, was administered to goats, it was found that in three to four hours the inorganic phosphorus in the milk was probably entirely composed of phosphorus which was present in the plasma after the administration. Casein showed a high activity of radio-phosphorus from which it appears that the phosphorus utilized in its synthesis is derived from the inorganic phosphorus of the plasma. From the rate of exchange of the phosphorus it was estimated that the time of formation of casein in the gland cells is about one hour. The ester phosphates are formed more slowly than is casein, and the milk phosphatides are extremely slow in formation.

Cohn & Greenberg (34) studied the disposition of the phosphorus of disodium hydrogen phosphate which was administered orally or by intraperitoneal injection to adult rats. Particular attention was paid to the rate and amount of excretion and the initial movements and storage of the phosphorus. It was found that the major disposition of the phosphate occurred within the first eight hours after administration. Absorption of the orally administered phosphate to animals fasted twenty-four hours was most rapid in the first two hours and was usually at an end in eight hours. About 30 to 40 per cent of the phosphate passed through into the feces unabsorbed. Of the injected or actually absorbed phosphorus, about 20 to 30 per cent was excreted in the urine within eight hours and about 3 per cent found its way back into the intestines.

The retention of phosphate per unit weight of fresh tissue decreased in the order: bone, liver, gastro-intestinal tract, heart, kidney, lungs, blood, muscle, skin, and brain (34). All of these tissues, except brain, showed a maximum of uptake in the first ten hours, followed by a prolonged, steadily diminishing loss. The turnover of phosphorus in brain was found to be very slow. The liver showed a much greater immediate uptake of phosphate when this was given by intraperitoneal injection than when it was given orally, which is the reverse of what was found with potassium given as potassium chloride. This is probably due, in part, to the fact that a considerable amount of the phosphorus is absorbed via the lacteals, and, in part, to the much slower rate of absorption of phosphate ion than of potassium ion.

The distribution of radio-phosphorus in the tissues of growing



chicks has been determined by Cook, Scott & Abelson (35). Phosphorus was deposited in all of the tissues examined, but principally in the skeleton and the musculature. All the soft tissues showed about the same degree of retention per gram of wet weight. In the time interval, four to sixty days, there occurred a marked shift of the phosphorus from muscle and intestine to bone. On the average the birds retained 77 per cent of the administered phosphorus and excreted 23 per cent in the sixty days' period.

A comparison of the phosphorus metabolism of normal and rachitic rats has been made by Dols, Jansen, Sizoo and coworkers (36, 37, 38). No significant difference was found between rachitic and normal rats in the absorption from or re-excretion of the labeled phosphorus into the gut. There was found in rachitic rats a significant increase of lipid phosphorus. From this the conclusion was drawn that rickets is associated with an increased formation or a decreased destruction of phospholipids. These workers, by means of radioactivity measurements and by taking radiograph photographs of the distribution of the active phosphorus, found that phosphorus metabolism is more intense in the bone of the rachitic chicken than in the bone of the normal chicken. Phosphorus metabolism was also more intense in the epiphysis than in the diaphysis.

Phospholipid metabolism by the use of radio-phosphorus has been studied by Artom and coworkers (39), Perlman, Ruben, Chaikoff and coworkers (40, 41, 42, 43), and by Hevesy and associates (44, 45, 46, 47, 48, 49). Labeled phospholipid was found in all of the tissues that were examined, and particularly high levels were found in the gastrointestinal tract, liver, and kidney. Two phases of metabolism, formation (or deposition) and utilization (or renewal) after maximum synthesis, have been discerned (40). The total amount of labeled phospholipid in the animal persisted for a long time, 80 per cent being present after fifty hours. Phospholipid could be synthesized by the intestines, and particularly by the liver, in the absence of fed fat, but was increased by the feeding of fat.

The major part of the phospholipid turnover by the gastro-intestinal tract is performed by the small intestine (41). The phospholipid turnover by the liver is not appreciably influenced by the removal of the gastro-intestinal tract and the kidneys. This is proof that the liver, as well as the small intestine is readily capable of synthesizing phospholipid.

Both in the laying and non-laying domestic fowl, the liver, kidney,

and small intestine showed the greatest radio-phospholipid activity. Egg-laying produced increased phospholipid activity in the liver, the blood, oviduct, and ovary. According to Hevesy & Hahn (49), the phosphatides formed in the liver and other organs are carried to the ovary by the blood plasma and but little phosphatide is formed in the ovary. After it leaves the ovary no further synthesis of phosphatide occurs in the egg. Evidence of phospholipid formation appeared in the egg yolk as early as five or six hours after the administration of the radio-phosphorus, the greatest deposition occurring in twelve hours.

In the chicken embryo, the phosphatides which accumulate as the embryo develops appear not to be derived from the phosphatides of the egg yolk (49). The embryo phosphatides therefore represent newly synthesized compounds. The phosphorus in the protein of the embryo also appears not to come from yolk phosphorus.

By experiments *in vitro*, Hahn & Hevesy (44) found that a small amount of phosphatide synthesis (0.3 mg. per cent) occurred by shaking blood with radioactive sodium phosphate. In this case the degree of synthesis was the same in normal and lipemic bloods. However, if a surviving liver was perfused with blood, a greater synthesis of phospholipids could easily be demonstrated, and, in the perfusion experiments, lipemic blood showed a greater phosphatide content than normal blood.

Experiments with radio-phosphorus have demonstrated that there is a continual, but slow turnover of the phospholipids of the brain (43, 46). After administration of labeled sodium phosphate to rats, phospholipid containing radio-phosphorus appeared in the brain within one hour and increased in amount up to two hundred hours. The rate of formation was the same in fed and fasted animals.

#### BLOOD CALCIUM AND PHOSPHORUS

*Serum calcium and protein.*—A better understanding of the relationship between the levels of serum calcium and protein has been reached with the development of the concept that the calcium proteinate of the blood plasma behaves as a weak electrolyte whose dissociation is governed by the law of mass action. The most important contribution to this has been made by McLean & Hastings (50) with the frog-heart method of estimating calcium ion concentrations. This technic leads to the conclusion that virtually all of the diffusible calcium of normal blood serum is ionized.

McLean & Hastings (50) calculated the calcium proteinate dissociation constant of serum to be  $pK_{Ca \text{ Prot.}} = 2.22$ . From the influence of the serum proteins upon the solubility of calcium carbonate, Weir & Hastings (51) obtained the value,  $pK_{Ca \text{ Prot.}} = 2.29$ . As is pointed out by McLean (52), a number of uncertain assumptions are involved in the numerical treatment of the calcium proteinate dissociation equation by McLean, Hastings and coworkers. The most important is the assumption that the value obtained from the titration of protein with base is a measure of the calcium-combining groups of the protein; or, in other words, that it represents the molal concentration of the protein with respect to its calcium combining groups. A second assumption is that the protein behaves toward calcium as if it were a series of divalent ions.

Greenberg, Larson & Tufts (53) and Greenberg & Larson (54, 55) have avoided these uncertainties by a graphical treatment of the mass law equation. A linear representation of the mass law is obtained by rearranging it into the form

$$\text{Total Protein}/[\text{Ca Prot.}] = 1/A + B/[\text{Ca}^{++}]$$

in which the concentration of total protein is expressed in grams per liter and  $[\text{Ca}^{++}]$  and  $[\text{Ca Prot.}]$  in molal concentrations.

In this equation,  $A$  represents the conversion factor required to express the protein content in terms of molal concentration, or, in other words, it represents the maximum molal calcium-combining capacity per gram of protein. The product of the constants  $A$  and  $B$  is equivalent to the equilibrium constant  $K$ . When the observed data obey the mass law, a straight line is obtained by plotting

$$\text{Total Protein}/[\text{Ca Prot.}] \text{ against } 1/[\text{Ca}^{++}].$$

The value of the constant  $A$  is given by the reciprocal of the point of intersection of the straight line on the  $\text{Total Protein}/[\text{Ca Prot.}]$  axis and the value of  $B$  is given by the slope of the line.

By this method of treatment the molal combining value of calcium per gram of protein (in normal serum) was calculated by Greenberg & Larson (54, 55) to be 0.0622 mM, and the mean value of the dissociation constant as  $pK_{Ca \text{ Prot.}} = 2.44$ . In their study, which was made chiefly on dog blood, extreme alterations in the calcium content of the blood were produced *in vivo* by injection of calcium salts and the administration of parathyroid extract. Augmentation, *in vitro*, was produced by the addition of calcium salts. The effect

of alterations in the serum protein content was also studied by means of plasmapheresis experiments.

Attempts to apply the mass law equation for the dissociation of calcium to pathological conditions and to species other than man have met with but qualitative success (56, 57, 58, 59, 60, 61). The explanation for this is that probably the composition of the serum proteins and their calcium-combining capacity are not so constant as has been assumed above.

Gutman & Gutman (60) studied the relation of the total serum calcium to the total serum protein and to the several protein fractions in the blood of 128 subjects, excluding cases with hyperphosphatemia and malnutritional hypoproteinemia. They found that the total calcium was directly proportional to the total protein in nephrotic and normal sera, but not in cases of hyperproteinemia, particularly if there was a hyperglobulinemia. It is suggested (60) that in hyperglobulinemia a form of globulin which binds very little calcium circulates in the blood stream.

Chu & Hastings (61) studied the calcium partition in concentrated human and horse serum in which the protein concentration was increased to 60 per cent above normal, and reached the conclusion that in serum with hyperproteinemia, the low combination of the calcium with the protein is due to the presence of abnormal proteins rather than to the failure of normal protein to combine with the predicted amount of calcium.

*State of the diffusible calcium.*—The view that the diffusible calcium of the blood serum is virtually all in the ionic form has not gone unchallenged (57, 59, 62, 63). Greenwald (63) has found that the presence of only small amounts of certain organic and inorganic anions produces large increases in the apparent solubility products of calcium sulfate, calcium carbonate, and calcium phosphate. It is suggested that calcium acetate, calcium sulfate, calcium carbonate, and calcium phosphate are only incompletely ionized, and that the calcium salts of  $\alpha$ - and  $\beta$ -glycerophosphoric, fumaric, maleic, and ascorbic acids have very low degrees of ionization. The concentrations of these acids in blood and tissues would have a marked effect on the ionization of calcium and the process of calcification. Cannan & Kilrick (64) have found support for Greenwald's views from the influence of divalent cations, including calcium, on the hydrogen-electrode titration curves of mono- and bivalent carboxylic acids.

*Colloidal calcium phosphate.*—Under certain special conditions, a

non-diffusible colloidal complex of calcium and phosphate is formed in the blood. The older literature concerning this phenomenon has been reviewed by Schmidt & Greenberg (1). McLean & Hinrichs (65), by means of direct observation of the calcium ion concentration with the frog-heart method, noted that when phosphate ion was added to serum, *in vitro*, the calcium ion concentration fell at a measurable rate, usually several hours being required for it to reach a steady state. The fall in calcium ion concentration was interpreted as being due to the formation of colloidal calcium phosphate.

On administering phosphate intravenously to dogs, in doses sufficient to cause tetany, there is rapid formation of the colloidal compound at the expense of the calcium ion and of the protein-bound calcium. A similar phenomenon is observed when large doses of phosphate are administered orally. Formation of colloidal calcium phosphate is presumed to follow when the product  $[Ca^{++}] [P]$  becomes  $> 3$ . The concentrations of the constituents are expressed in millimols per liter.

According to McLean & Hinrichs (65), colloidal calcium phosphate is rapidly removed from the blood. Evidence for this is that preformed colloidal calcium phosphate suspended in serum is rapidly removed from the circulation when administered intravenously to either normal or thyroparathyroidectomized dogs.

Gersh (66, 67), using the freezing-drying technic and staining with silver and alizarine, has observed that colloidal calcium phosphate is phagocytized by the macrophages of the liver and spleen. It is rapidly removed from the blood plasma and its existence, even when present in massive amounts, is rather transitory. When the calcium and phosphate concentrations of the blood plasma are reduced, the phagocytized calcium and phosphate are returned to the blood. The colloidal calcium phosphate that has been taken up from the blood stream by phagocytes appears as brown or black granules in the cytoplasm of the cells that have been treated with a 60 per cent solution of silver nitrate saturated with silver phosphate and silver chloride.

*Cerebrospinal fluid and serum calcium.*—The relation between the calcium of the cerebrospinal fluid and plasma has been restudied by Cameron & Moorhouse (68). Using the procedure of continuous open drainage from the cisterna magna they found that the concentration of calcium in the cerebrospinal fluid does not respond readily to changes in the plasma calcium content. Only when the concentration of blood calcium was normal did the level of calcium in the cerebro-

spinal fluid correspond with the level of the diffusible serum calcium as determined by other methods.

*Milk.*—Cox & Mueller (69) have reported on the mineral composition of the milk of normal rats.

*Pregnancy.*—Mull (70) found that the calcium and phosphorus concentrations were higher in cord blood than in the maternal blood and varied according to the calcium and phosphorus levels of the maternal blood. Denzer, Reiner & Vogel (71) and Bakwin (72) observed that there is a constant relationship between the cord and postnatal blood. There is a tendency for the serum calcium to assume a lower value than that of the cord blood during the first four days of life, and then to rise toward the prenatal level during the next five days.

The serum calcium level tends to be low in the later stages of pregnancy (73, 74), while the inorganic phosphorus and phosphatase remain at their normal levels. The reduced serum calcium content is correlated with a negative calcium balance (74).

*Pathological conditions.*—No abnormalities were found in the concentration of calcium ion, inorganic phosphorus, or phosphatase in allergic diseases (75) and in urinary lithiasis (76). Subjects suffering from Paget's disease of bone were found to show a smaller calcium rise upon intravenous injection of calcium gluconate than normal persons (77). Norman (78) is of the opinion that certain angiospastic conditions are characterized by a low serum calcium level and that calcium therapy is of value in their treatment. Serum calcium and phosphorus values are usually normal in marble bone disease (osteopetrosis) (79, 80).

The development of rickets was found to be associated with decreases first of inorganic phosphorus and adenosinetriphosphate and then of diphosphoglyceric acid in the blood corpuscles (81). In healing, the phosphorus fractions increased in the reverse order.

In spite of abundant sunshine, about 50 per cent of Egyptian infants are rachitic and exhibit low blood phosphorus levels during the winter and spring (82). Dunkelmann (83) observed a low level of inorganic phosphorus in the blood of children with pneumonia. The blood phosphorus was increased and the clinical course and prognosis were stated to be improved by vitamin-D therapy.

*Influence of hormones.*—Except for the work of Pfeiffer & Gardner (87) it may be concluded that administration of the estrogenic hormones of the gonads is without effect on the blood calcium level

(84, 85, 86). Ovariectomy is reported to increase the serum calcium concentration (88). The question of whether the anterior hypophysis exerts parathyreotropic activity is still unsettled. Hypophysectomy had no effect on the concentration of serum calcium (89), but, on the other hand, injection of an alkaline extract of the anterior hypophysis produced a significant elevation in the serum calcium level (90).

According to Deobald, Christiansen & Hart (91), a high blood calcium is not an index of egg production in the hen. Laskowski (92) found that the gonadotropic hormone of the pituitary caused enormous increases in the concentration of phospho-protein and other phosphorus fractions of the blood plasma of resting hens. The hormones of the gonads obtained from the urine had no influence on the blood phosphorus.

*Blood coagulation.*—As estimated with the frog-heart method, lowering the calcium ion concentration of blood plasma to values less than 1.25 mM per liter results in an increased coagulation time (93, 94). The minimal concentration of calcium ions at which coagulation occurs is about 0.35 mM for human and 0.24 mM for dog plasma. Ferguson (95) postulates that a calcium-cephalin-prothrombin compound acts as an intermediate in thrombin formation.

*Effects of calcium salts.*—Lands & Woodard (96) have found that injected calcium chloride leaves the blood stream rapidly due to an exchange between the blood stream and the tissue spaces. About 70 per cent disappeared from the blood stream within five minutes and this occurred in the absence of bony tissues, but removal of the large intestine reduced the rapidity with which the calcium left the blood stream. Seekles (97) found that the rate of disappearance of injected calcium from the blood stream was proportional to the calcium excess.

Moraczewski & Jankowski (98) state that increasing the blood calcium level by oral or intravenous administration of calcium salts, or by parathyroid extract, increases the concentrations of fatty acid and cholesterol in the blood.

The use of calcium and the choice of a calcium salt in medication have been discussed by Aub (99). Increase of the calcium stores of the bones is best accomplished by drinking large quantities of milk. If milk cannot be tolerated, calcium gluconate and calcium lactate are most valuable. Calcium gluconate may be injected either intramuscularly or intravenously without danger of thrombosis or of tissue sloughing. It is of value in tetany, spasmophilia, and in other spasms of smooth muscle, particularly in lead colic. Gisselsson & Sylvan



(100) have observed that the administration of calcium gluconate or levulinate fails to produce an alkalosis as would be expected from the fact that these salts leave an alkaline ash.

*Liver injury.*—Some years ago it was observed that in the presence of liver injury produced by phosphorus or hydrazine poisoning, parathyroid administration did not cause its characteristic rise in the serum calcium level of dogs (101). Lederer & Crandall (102) found that Eck fistula dogs exhibit a low level of serum calcium in the fasting state, a lessened rise in serum calcium following the oral administration of calcium lactate or calcium gluconate, an abnormally rapid removal of calcium from the blood after intravenous injection of calcium chloride, and a decreased effectiveness of parathormone in mobilizing calcium. The conclusions drawn were that these animals either suffer from a calcium deficiency which is secondary to a decrease in bile excretion, or that the observed effects are due to some endogenous relation of the liver to calcium metabolism. Gastrectomized dogs showed a normal absorption of orally administered calcium salts.

#### NEUROLOGICAL EFFECTS

The relationship of the elements calcium, magnesium, and phosphorus to nervous activity, particularly to the syndrome of tetany, has received considerable attention.

Much of this work has been done on the rat. This animal is resistant to the onset of tetany even following the removal of the parathyroid glands. This resistance increases as the animal attains its full growth. Jones (103) found that weaned young rats will experience tetany within a day or two following parathyroidectomy if they are fasted for twenty-four hours immediately following removal of the parathyroids. A diet adequate in calcium and phosphorus with a calcium : phosphorus ratio of 1.7 protected the parathyroidectomized rats against tetany.

Bodansky & Cooke (104) observed the onset of tetany in adult female rats only when the additional strain of pregnancy was added to the effect of thyroparathyroidectomy. A large percentage of their experimental animals died at term from convulsive seizures either before or after the onset of labor. The serum calcium level in these animals was found to be between 4 and 6.5 mg. per cent. Greenberg, Boelter & Knopf (105) obtained tetany in parathyroidectomized rats of weaning ages only if they were fed on a diet low in calcium.



Patras, Galapeaux & Templeton (106) state that calcium hydroxide is superior to calcium chloride as a dietary salt for the prevention of tetany during the first forty-eight hours following thyroparathyroidectomy. They also observed that a defective salt intake prior to the operation increased and the injection of ammonium chloride decreased the mortality and morbidity.

Rats with intact parathyroids, reared on a diet very low in calcium but with plenty of vitamin D, did not develop tetany even though the blood serum calcium was reduced to the low levels of between 4.4 and 6.6 mg. per cent (105). After six weeks or more on this diet, the rats collapsed if they were subjected to short, mild, galvanic shocks from an induction coil. The animals responded poorly and sluggishly to all stimuli. The body surface became anesthetic and there was a paralytic foot drop of one or both hind limbs. Hemorrhage occurred in various parts of the central nervous system, in the lungs, gastrointestinal tract, bladder, and muscle. The nervous involvement was probably a consequence of the hemorrhage in the nervous system. The same condition developed spontaneously and in a much more severe form in animals maintained for longer than nine weeks on the low calcium diet. If death did not occur, the anesthesia and paralysis disappeared more or less completely.

It has been suspected for some time that tetany is not the result of reduced levels of blood and body fluid calcium alone (1, 65). In the rat, tetany occurs when a low serum calcium is associated with hypoparathyroidism or vitamin-D deficiency (107).

Possibly, rapid fluctuations in the concentration of ionic calcium in the body fluids may be of importance in inducing tetany; after a time, the organism may adjust itself to a continuously low level. If the concentration of inorganic serum phosphorus was kept low by the addition of aluminum acetate to the diet, a normal level of blood calcium was maintained, and parathyroidectomized rats were protected against tetany (108). However, if the serum phosphorus content increased either as a result of tissue catabolism or an optimum diet, the calcium level fell and tetany developed. This also held true for rachitic rats (1). Hastings & Lees (109) produced a typical tetanic syndrome in dogs by reinjecting calcium-depleted cerebrospinal fluid, or by injecting small amounts of sodium citrate into the cisterna magna of anesthetized dogs. The seizures could be overcome by restoring the calcium ion concentration to normal. Bakwin (110) states that the tetany of new-born children is characterized by an

abrupt fall in the level of the serum calcium within a few hours after birth. There is a low content of phosphorus in the urine and a hyper-responsiveness to the ingestion of phosphates. He believes this form of tetany is caused by hypoactivity of the parathyroids.

Dogs with latent tetany seven to ten months after thyroparathyroidectomy may have a blood calcium level as low as it is in acute tetany (111). The inorganic serum phosphorus also may be as high in latent as it is in acute tetany. The beneficial action of insulin in tetany, according to Mathieu (111), is not due to an increase in the serum calcium. The use of high doses of vitamin D in the treatment of parathyroid tetany continues to receive favorable support (112, 113).

The neurological mechanism of tetany has been studied by West (114). His experiments support the hypothesis that there is an involvement of the myoneural junction. In the dog the motor manifestations of parathyroid tetany may be tonic, clonic, or fibrillary. After section of the peripheral nerves, clonic and fibrillary tetany occurred in muscles up to four hours after denervation. Fibrillary tetany, but not clonic tetany, occurred for at least twenty-four hours after denervation. West observed all three forms of tetany in parathyroid-ectomized dogs after trans-section of the spinal cord, for many days after the operation.

In tetany due to magnesium deprivation in the rat, Greenberg & Tufts (115) have suggested that the mid-brain is involved because the sound of an air jet is the best stimulus and a galvanic stimulus does not easily induce attacks. Furthermore, small doses of curare did not prevent attacks, but amytal did. These animals also showed an increased sensitivity to the drug picrotoxin which acts upon the mid-brain. Tetany was induced by electrical stimulation but not by the air-jet sound in rats with rapidly healing rickets and rats on a low calcium, low vitamin-D diet. The character of these tetanic seizures appeared to be identical with those of magnesium tetany (107). Heppel & Schmidt (116) induced attacks of tetany in rats reared on a diet low in potassium but containing rubidium; the sound from an air jet was used as the inducing agent.

#### METABOLISM

*Dietary requirements.*—From a statistical evaluation of balance studies, Leitch (8) has estimated that the maintenance requirements of children for calcium is between 0.8 and 1.0 gm. per day for the

ages between six months and fifteen years. His estimate for adults is 0.55 gm. per day, which is about 20 per cent higher than the well-known value set by Sherman. In Leitch's estimation, the amount of milk consumed by breast-fed infants is frequently inadequate to provide sufficient calcium to maintain the correct body composition. The calcium and phosphorus intake of pregnant women should be between 1.4 and 2.0 gm. per day according to Morgan (7). This requirement should be met by the ingestion of milk and other calcium-rich foods rather than by the ingestion of calcium salts.

The calcium, phosphorus, and magnesium requirements of farm animals for growth, pregnancy, lactation, and egg production have received detailed consideration (5, 6, 117, 118).

*Self selection.*—Richter and coworkers (119, 120, 121) observed a definite ability of rats to select an adequate diet from a number of purified foods. The animals were found to possess a special appetite for the essential mineral elements, including calcium. The appetite for calcium is increased during pregnancy and in parathyroid deficiency.

*Availability of calcium in vegetables.*—It has been known for some time that the calcium in spinach and certain other green vegetables is very poorly utilized (122, 123, 124). The most probable explanation for this lies in the high oxalic acid content of the vegetables. Fairbanks & Mitchell (123) found that the rat is totally unable to utilize calcium oxalate.

*Optimum calcium intake.*—From a study of the enrichment of an already adequate diet, Sherman, Campbell & Rice (125) found that extra calcium, as well as vitamin A and riboflavin, expedited growth and development and increased the life span. Rats fed at a level of 0.64 to 0.8 per cent calcium attained a percentage of body calcium at one month of age that would have required five to six months to attain on a 0.2 per cent calcium diet (126). The experiments indicated that the higher calcium content is advantageous throughout life. The addition of calcium lactate to a diet typical of South India, which consists largely of rice, led to an increased growth rate in rats (127). Improvement observed in a typical British diet, by the addition of milk and greens, was attributed to the increased calcium and phosphorus content (128).

Mendel and coworkers (129, 130) found that the Osborne-Mendel salt mixture in a synthetic diet afforded better growth and a higher bone ash than did the McCollum, Steenbock, or Sure salt mixtures.

Even so, the Osborne-Mendel salt mixture was improved by approximately doubling the ratio of calcium to the other inorganic elements of the salt mixture. On the basis of the accumulation of calcium in the carcass, Campbell (131) states that calcium carbonate supplies the calcium requirements of rats as well as does milk.

In the opinion of Rottensten (132), the degree of calcium and phosphorus saturation of the tissues has an important influence on the efficiency of utilization of dietary calcium. Because of this a positive calcium and phosphorus balance may not necessarily be proof of an adequacy of intake, but instead may only be a reflection of the level of the previous mineral nutrition.

*Absorption of phosphorus.*—The rate of absorption of sodium phosphate, according to Laskowski (133), is proportional to its concentration in the intestines. The absorption is more rapid in the upper than in the lower part of the small intestine. The organic phosphorylated compounds have to be hydrolyzed by phosphatases before their contained phosphate can be absorbed. It was found that the parathyroid hormone enhanced the absorption of phosphates, but that calciferol exerted no such influence.

*Fecal excretion.*—According to the results of observations on human subjects with isolated colons, little or no calcium is excreted into this organ (134, 135). On the other hand, from the chemical composition of rabbit pellets at different levels of the large intestine, Cowell (136) concluded that calcium can be excreted by the upper part of the colon of rabbits.

According to Westerlund (137), the fecal excretion of calcium in milch cows varies in direct proportion to the consumption of calcium and protein and inversely as the phosphorus consumption and the amount of milk calcium. Roughage in the form of regenerated cellulose produced no significant increase in the fecal calcium and phosphorus even when it was fed at as high a level as 25 per cent of the diet (138); opposed to this observation is the work of Westerlund (137). According to Robertson (139) diets low in calcium may be a cause of a hypotonic colon and intestinal stasis.

Intravenous injection of calcium or phosphate salts has but little influence on the fecal excretion of either calcium or phosphorus (140, 144).

*Calcium balance.*—Aub, Tibbetts & McLean (141) have studied the influence of the parathyroid hormone, urea, sodium chloride, fat, and intestinal activity upon the calcium balance. It was found that

the excessive parathyroid excretion in hyperparathyroidism, voluntary constipation or diarrhea, the ingestion of an organic solvent like urea, the ingestion of agar, of large amounts of fat or of sodium chloride have but little influence on the absorption or excretion of calcium.

*Influence of vitamin D.*—In normal young women, Maxwell (142) found that vitamin D, either as cod-liver oil or as irradiated milk, had no effect on the calcium retention. In Chinese women suffering from osteomalacia (143), vitamin-D administration was most efficacious in producing a positive calcium and phosphorus balance by reducing the fecal loss of calcium. Addition of calcium to the diet alone was not very efficacious. In these cases the negative calcium balance was due mainly to loss of calcium in the stools rather than through its excretion into the milk. The yield of breast milk was reduced by a low calcium intake or vitamin-D deficiency. In a case of rickets resistant to vitamin-D therapy, Albright & Sulkowitch (140) found that large amounts of vitamin D decreased the fecal phosphorus and calcium excretion and increased the urinary excretion of calcium but not of phosphorus.

*Mode of action of vitamin D.*—Current work supports the hypothesis that vitamin D acts by producing an increased absorption of calcium from the gut and by increasing the excretion of phosphorus into the urine (140, 144, 145, 146, 147). Vitamin-D deficient rats excreted about five times the normal amount of fecal calcium. Vitamin-D deficiency did not cause an increase in the fecal excretion of phosphorus. On a low phosphorus diet, lack of vitamin D led to a reduction in the amount of calcium absorbed by rats. Vitamin-D deficiency did not retard the intestinal absorption of phosphorus from inorganic phosphate, glycerophosphate, or casein in rats fed a low calcium diet (144).

Innes & Nicolaysen (145) have demonstrated that the resistance of rats to rickets is not due to a better utilization of the phosphorus from foods containing phytin because of bacterial action in the large intestine. The assimilation of calcium and phosphorus by rats, with and without a caecum, is the same either in the presence or absence of vitamin D. Vitamin D does not affect the absorption of calcium by altering the pH of the intestinal tract (146).

Both dihydrotachysterol and vitamin D have the same fundamental action of increasing calcium absorption from the intestines and phosphorus excretion into the urine (147). However, dihydrotachysterol has a greater effect on the phosphorus excretion than on

the calcium absorption which may explain its failure to be anti-rachitic.

Nicolaysen (144) has calculated that a rachitic rat requires 7.5 mg. of calcium and 4.5 mg. of phosphorus per day. This is about one-fifth the amount absorbed by normal rats.

*Effects of sugars.*—According to Outhouse, Smith & Twomey (148), lactose is not so efficient as cod-liver oil, but is much more effective than starch or sucrose in producing good retentions of calcium, magnesium, and phosphorus, and high bone-ash values in the rat. In the dog, French & Cowgill (149) found that lactose favors the utilization of calcium and phosphorus in immature but not adult animals. This is accomplished by a diminished excretion of fecal calcium.

*Parathyroids in thyroid dysfunction.*—The relation of the parathyroids to the calcium and phosphorus balances in hyperthyroidism remains to be cleared up. According to Cope & Donaldson (150), the calcium and phosphorus changes in a case of coexistent hypoparathyroidism and hyperthyroidism were due to alteration in thyroid function and not to the hypoparathyroidism. Because there is no correlation between the calcium balance and the basal metabolic rate, and because treatment with Lugol's solution has no effect, Hansman & Carr Fraser (151) believe that the negative calcium and phosphorus balances in hyperthyroidism are due to an associated parathyroid dysfunction.

*Mode of action of the parathyroid hormone.*—The hypothesis that the parathyroid hormone acts directly on the kidney to produce an increase of phosphate excretion has received further support (152, 153, 154).

## MAGNESIUM

Considerable progress is being made in establishing the functions of magnesium in the economy of the animal organism.

*Blood magnesium.*—Eveleth (155) found high magnesium values in the blood plasma and corpuscles of rodents and swine, while, on the contrary, the corpuscle-magnesium contents of the ruminants were characteristically low, often lower than the plasma-magnesium level. These differences did not appear to be related to differences in the food habits.

Hirschfelder & Haury (156) state that the concentration of the plasma magnesium is frequently low, while the cerebrospinal fluid

magnesium remains normal during convulsive seizures in cases of essential epilepsy. Greenberg & Aird (157) found no significant variations from the normal in the magnesium content of the plasma, corpuscles, or cerebrospinal fluid of epileptic subjects.

The blood-magnesium levels have been examined in a number of pathological conditions. Increases in the blood-magnesium level were found in kidney diseases in which there was an obvious change in renal function (158, 159).

Contrary to previous reports, Borgström (160) found no change in the serum-magnesium level of rabbits receiving prolan injections. Suomalainen (161) has observed that the serum-magnesium content of the hedgehog increases from 3.2 mg. per cent before hibernation to 5.4 mg. per cent during hibernation, while the calcium content remains unchanged. He postulates that the increased serum magnesium has an anesthetic effect. A similar change in magnesium with hibernation has been observed in *Helix pomatia* (162).

*Magnesium balance.*—From a study of the magnesium balance of human subjects in health and in certain diseased states, Tibbetts & Aub (163) concluded that calcium, magnesium, and phosphorus may be independently metabolized and do not necessarily respond as a group. Using three-day collection periods, these authors found that on a low calcium neutral diet an essentially positive balance was maintained by hospital patients on an intake of 220 mg. per day. Medical students pursuing their normal activities stored magnesium when the intake was 300 mg. per day. On the levels of intake given above, about one-third of the magnesium was excreted in the urine and two-thirds in the feces; this distribution in the excreta resembles that of calcium.

The ingestion of extra magnesium in the form of magnesium lactate did not cause an extra storage of magnesium in normal subjects. The extra magnesium was excreted mainly by the intestine, as might be expected, but the urinary excretion was also increased to as much as six times the control level, indicating that the lack of storage was not due to failure of absorption.

Little difference in the magnesium balance from that of normal controls was found in hyperparathyroidism, exophthalmic goiter, Cushing's disease, Addison's disease, steatorrhea, and pituitary basophilism in spite of the marked changes in calcium metabolism which are found in these conditions. Following parathyroidectomy, there is a temporary fall in the magnesium excretion, particularly into the



urine. The excretion returns to the preoperative level within a few months after the operation. The administration of parathyroid extract produces a temporary increase in the excretion of magnesium into the urine.

The ingestion of extra magnesium, as magnesium lactate, increased the urinary calcium in normal subjects and also increased the calcium drain in hyperparathyroidism. Magnesium lactate also accentuated the extra urinary excretion of calcium caused by ammonium chloride. The loss in calcium produced by these agents could be checked by a large intake of sodium acid phosphate.

An acid diet tended to increase the urinary magnesium without appreciably altering the fecal excretion. The extra urinary magnesium is probably derived from the soft tissues and not from the skeleton.

*Magnesium deprivation.*—The dramatic findings of Kruse, Orent & McCollum (164) have aroused widespread interest in the effects of magnesium deficiency. Recent work has shown that far-reaching pathological changes are produced by this condition. According to Tufts & Greenberg (165), there are two phases of physical change in magnesium deficiency. In the first place the chief manifestations are vasodilatation, hyperemia, and hyperexcitability, and in the second, malnutrition, cachexia, and kidney damage. These manifestations have been observed in varying degrees by all investigators. The reports on the extent of the pathological involvement of the skin and certain other organs have not been so consistent.

Because it is difficult to obtain adequate vitamin supplements low in magnesium, it seems probable that deficiencies in various members of the vitamin-B complex are a complicating factor and are responsible, in part, for certain of the discordant observations that have been reported, particularly those involving the epidermal structures, the gastro-intestinal tract, and the liver. The alcoholic extract of yeast (164, 172), commonly employed as a vitamin-B supplement in this work, is apparently low in both riboflavin and vitamin B<sub>6</sub>. One group of experimenters (167) either gave no vitamin-B supplement or only 10 mg. of a fullers' earth adsorbate of yeast. It should also be noted that where the vitamin supplement is mixed with the basal ration, the experimental animals may obtain insufficient vitamins because of reduced food consumption.

However, since in the work on magnesium deprivation the controls are reported to be free from the pathological changes found in



the test animals, it would appear that certain of the effects are due to a synergistic relation between lack of magnesium and lack of certain members of the vitamin-B complex.

Watchorn & McCance (166) observed loss of fur and varying degrees of dermatitis on a diet containing 4 mg. per cent of magnesium. Schrader, Prickett & Salmon (167) obtained a severe erythema followed by hemorrhagic purpura and then eschar formation on a diet (magnesium content not stated) of acid-extracted yellow corn, wheat middlings, casein, and a supplementary salt mixture.<sup>1</sup> The animals of Tufts & Greenberg (165) developed a greasy, sticky coat and the tails, paws, and ears became encrusted with a sticky brownish material which was probably an exudate of a keratin protein and lipoids, as described by Watchorn & McCance (166). Neither dermatitis nor localized loss of hair was observed. In later studies, Greenberg, Boelter & Tufts (168) found that the onset of the skin defects could be retarded but not eliminated entirely. On a diet which contained, per 100 gm. of food, 1.6 mg. of magnesium (supplemented with 0.6 mg. of crystalline thiamin hydrochloride, 0.5 mg. of crystalline riboflavin, and 3 cc. of an 85 per cent alcoholic extract of a rice-bran preparation), epidermal abnormalities did not begin to develop until thirty-five days had elapsed.

Watchorn & McCance (166) observed that their piebald but not their albino rats were prone to have hemorrhages in the gastro-intestinal and urinary tracts. Occult blood in the stools, and hematuria, were common findings. These authors observed a slight degree of kidney damage without albuminuria and no evidence of liver damage. The intestinal tracts of the experimental animals of Schrader, Prickett & Salmon (167) were mildly atonic and congested and about one-third of the animals evidenced degenerative changes in the liver.

The best established pathological changes in magnesium deficiency are those occurring in the kidney, heart, teeth, and bones (164, 166, 168, 169, 172, 173).

Greenberg, Lucia & Tufts (169), with lower dietary levels of magnesium than the above workers, did not find liver damage or hemorrhage in the gastro-intestinal or urinary tracts. Albuminuria and the development of a nephrotic type of kidney disease were the most pronounced findings in their animals. There was an increase in

<sup>1</sup> The report by these investigators, founded on experiments with the same basic diet, that potassium deficiency leads to profound gastro-intestinal lesions, has not been substantiated by the work of Heppel & Schmidt (116).

the volume of urine and in the excretion of protein, but blood or casts were not evident. The serum-protein content progressively diminished and finally reached a level at which edema developed. The pathological changes in the kidney consisted of degenerative changes in the tubules and calcification, mainly in the cortico-medullary zone and in the pyramids.

Swanson, Storwick & Smith (170) observed kidney damage in rats reared on a diet with a very low mineral content. It is probable that a deficiency of magnesium was an important etiological factor in their findings. With high dietary calcium levels of between 1 and 2.4 per cent, Cunningham & Cunningham (171) obtained bladder calculi and kidney damage when the magnesium level of the ration was as much as 17 mg. per cent. Calculus formation could be prevented by the addition of 0.76 per cent magnesium carbonate or of 2 per cent sodium phosphate to the ration. The authors attribute calculus formation to the effect of a high serum calcium associated with a low serum magnesium and an alkaline urine.

In the calf, degeneration of the blood vascular system is more characteristic of magnesium deficiency than is kidney damage. Moore, Hallman & Sholl (172) have observed extensive calcification in the yellow elastic tissue of the endocardium and of the intima of the large arteries and veins in calves made magnesium deficient by being reared on milk. Myocardial degeneration and polyblastic infiltration of wandering cells have been observed in magnesium-deficient rats (173).

More uniform results have been obtained concerning the chemical changes that occur in magnesium deficiency. There is a reduction in the blood magnesium, in the percentage of whole body magnesium and bone magnesium, and little or no reduction in the magnesium contents of the soft tissues (164, 165, 166).

According to Tufts & Greenberg (165), the magnesium level of the plasma and red corpuscles begins to drop within a few days after the animals are placed on the low magnesium regimen. The plasma magnesium drops sharply to levels of less than 1 mg. per cent and then rises again to a peak shortly after the onset of hyperexcitability. It falls off again during the second phase, at a relatively slow rate. The magnesium content of the red corpuscles is reduced to about half the normal value and remains fairly constant at the lower level during the further course of the deficiency.

No difference from the normal level has been found in the phos-

phatase content of the blood plasma, kidney, or bone (166). The magnesium content of the bone is decreased, the reduction being greater the lower the magnesium content of the diet. The teeth also show a reduced magnesium content (164, 166).

The calcium contents of the soft tissues are increased, the increase being from 50 to 100 per cent in the heart and the muscle, and as much as 15-fold in the kidney. The percentage of the whole body magnesium is reduced to about two-thirds the normal level and the percentage of the body calcium is increased to about one-third above the normal value.

There is little retardation in growth in the first phase, and a marked retardation in growth in the second phase of the magnesium deficiency. The retarded growth appears to be due mainly to a lowered food intake. At least 75 per cent of the weight difference between controls and deficient animals can be accounted for as being due to this. Magnesium-deficient animals show no significant change in the water content of the soft tissues (165, 166).

An increase in the calcium content of the diet increases the severity of the magnesium deficiency and raises the amount in the diet required to met the needs of the animal (165). A diet containing 5 mg. of magnesium per 100 grams of food or an intake of approximately 0.4 mg. per 100 grams of body weight per day is the approximate minimum requirement for good growth with ordinary levels of dietary calcium. Females on this diet gave birth to young of normal weight and normal body-magnesium content. However, these young developed symptoms of magnesium deficiency during lactation, indicating that the mother's milk on this level of magnesium intake is deficient in magnesium. There was no increase, and in some instances there was even a loss, in the percentage of body magnesium of the young rats during lactation.

Hoobler, Kruse & McCollum (174) measured the ultrafilterable magnesium in magnesium-deficient dogs and found extremely low levels of diffusible magnesium but nearly normal levels of diffusible calcium. In their estimation, the tetanic syndrome of magnesium deficiency is closely associated with the extremely low concentration of magnesium ions in the blood.

In dogs maintained on a diet containing 1.7 mg. of magnesium per day, the urinary excretion of magnesium decreased from about 8 to 2 mg. per day over a period of thirty days, while the fecal excretion remained relatively constant at a level of between 6 to 8 mg. of

magnesium per day (175). Upon parenteral injection of magnesium, 70 to 90 per cent appeared in the urine, and the intestinal elimination remained unaffected. Very little magnesium was excreted through Thiery-Vella fistulas in dogs in which the colon and ileum were anastomosed to the rectum.

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## HORMONES

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This chapter is a survey of recent developments in studies of the chemistry and physiological action of hormones. The steroid group (sex and adrenocortical hormones), whose chemical composition is known, and extracts of the anterior pituitary, the active agents of which have not yet been determined, have been most widely studied during this period. There is a growing tendency to analyze the physiological action of hormones, rather than to describe it in a summary way. For example, whereas early workers spoke only of growth or masculinization the present tendency is to seek out the underlying biochemical and cytological processes for detailed analysis. The whole action of any given hormone is, in reality, the sum of single effects, which in turn are dependent not only upon the substance producing them, but also upon many other factors such as the quantity of hormone administered.

A tremendous amount of work has been done on this subject in the last year. The literature is four to six times greater than could be dealt with here. It has been necessary, therefore, to omit many important observations (1).

## ADENO-HYPOPHYSIS

More than ever before, the hypophysis is now recognized as the source and target of active substances and of various factors which determine their effects. Agreement of workers to adhere to a standard description of their preparation of active extracts and of common experimental procedures would contribute greatly to further advances in this field.

## HISTOLOGY AND BIOLOGY

The significance of basophilic and acidophilic cells of the hypophysis has been studied (2, 3). In pigeons, acidophils act on growth, crop glands, and adrenals, and basophils on the gonads and thyroids (4). In other species basophils, too, act on the adrenals. In man 24

per cent acidophils were found in infancy (body length less than 55 cm.), and 38 per cent later (5). In diabetes there is a lack of acidophils (6). Pancreatectomized cats have large hypophyses without acidophils (7). A case of schizophrenia, dead as a result of insulin-shock therapy, showed hyperacidophilism (8). The peripheral (acidophilic) zone of beef hypophysis inhibits metamorphosis of tadpoles and stimulates their growth, while the central (basophilic) cells stimulate thyroids and metamorphosis; results in mice, rats, and pigeons were similar (9).

Skin mucification, poor calcification, hypertrophy of proliferating cartilage, and epiphysiolysis occurred in larval salamanders after implantation of pituitaries (10). Thyroidectomy or hyperthyroidism alters the hypophysis (11). The effect of thyroidectomy on the hypophysis is similar to, but somewhat different from that of castration. Parathyroidectomy and calcium chloride injections produced acidophils in the hypophysis, and parathormone produced basophilism (12); an inquiry into the influence of phosphorus is needed. In lactating rats no castration cells and no luteinisation of intrarenally implanted ovaries were obtained until weaning (13). The hypophysis of *Xenopus laevis* (South African) had a gonadotropic action when implanted into infantile mice (14). Human hypophyses had thyreotropic and gonadotropic effects in guinea pigs. The "follicle stimulating" potency of children's hypophyses was great, that of climacterics and adults medium, and that of women in late pregnancy almost negligible (15). Alkaline extracts of human pituitaries increased the weights of the ovaries and the uterus of immature rats; similar extracts from the pituitaries of children were of low potency, those of adults medium, and those of old men and women and of dysmenorrhoeic women were of high potency (16). The "neurocrinie" and innervation of the pituitary by the "thirteenth" cranial nerve indicate the pituitary-hypothalamic relationship (17).

As may be concluded from animal experiments and from many human specimens, metabolic effects of the pituitary are transmitted in part by way of the central nervous system (18). Severance of the pituitary stalk in rats and rabbits leads to gradual atrophy of the ovaries; luteinisation after copulation and ovulation after the application of electrical stimuli to the brain fail to occur. Even in such animals, however, the pituitary is enlarged by estrone; castration cells fail to develop; gonadotropic extracts from urine are as active as in normal animals; and estrone stimulates luteinisation (19).

Infundibular and hypothalamic centers regulate the hyper- and hypothyreotropic responses of the hypophysis to temperature. In human urine thyreotropic activity may be detected after exposure of the subject to cold (20). The nervous regulation of pituitary function has been reviewed recently by Haterius (21).

The pituitaries of hereditary dwarf mice lack acidophils. The thymus, parathyroids, and pancreas show interesting changes from the normal. Growth and vitality of such mice are restored by crude pituitary extracts, as well as by prolactin, thyreotropic hormone, or thyroxin (22). The males often show phimosis as do hypophysectomized rats (unpublished observations); and in females Graafian follicles and corpora lutea fail to develop. The pituitaries of these mice show gonadotropic activity when implanted into normal immature mice (23). It is claimed that the pituitary stimulates or inhibits the reticuloendothelial system (restropic factor) (24). Studies of the importance of physical factors (25 to 31) and x-rays (32 to 35) to pituitary function have been made.

The effects of hypophysectomy have been further studied. In young ferrets hypophysectomy inhibited skeletal and skull development and lowered the calcium content of the bone (36). Atrophy of the gonads occurred in male *citellus* (37). Dogs survived 2.5 years without skeletal growth, but their brains, livers, and lungs were small, and other viscera and endocrines much underdeveloped. Mental infantilism was noticeable (38). Hypophysectomized baby pigs died after weaning (39). Mice showed nuclear and protoplasmic atrophy in all the cells of the body, although the total number of cells was not conspicuously changed. Their skin became parakeratotic (40). Hypophysectomy of rats caused atrophy of the thymus, which could be restored by pituitary extracts (41). Their muscular power in the "swimming test" was low, and was not changed by cortical hormones or a salt diet (42). The gastrocnemii of hypophysectomized rats were quickly fatigued by three electrical stimuli per second (43). Spontaneous activity was depressed but could be restored by corticotropic extracts of the pituitary (44). Their stomachs contained hydrochloric acid after feeding (45), which is significant in view of the rôle of the hypophysis in haemopoiesis and haemoclasia.

The reticulocytes disappear from the blood of hypophysectomized rats (46 to 49). Therefore, a primary haemoclastic effect of the hypophysis has been postulated. This hypothesis is supported by the added facts that hypophysectomized rats made anemic by bleeding or

phenylhydrazine poisoning developed a reticulocytosis, as do normal rats, and because potent pituitary extracts decreased the number of red cells and indirectly produced reticulocytosis. Disturbance of location and fat transport after phosphorus and carbon tetrachloride poisoning was studied (50).

Further studies have been made of the effect of sex hormones upon the pituitary. The thymus is enlarged in castrates. Estrogens in such animals tend to enlarge the pituitary (51), to decrease the thymus, and to inhibit growth, but the gonadotropic potency remains virtually unchanged (52). Giant pituitary adenomata were produced in rats by long and intensive treatment with estrogens. Dwarfism and gonad atrophy resulted, but there was no visible damage to the thyroid, adrenals, and implanted tumor tissue. In birds there was a loss of plumage and spurs (!), and haemopoietic marrow was replaced by bone (53). Pituitary implants from rats, which had been pretreated for eight days with 43  $\mu$ g. of testosterone propionate daily, increased the weight of the uterus in immature mice (54). Implants from rats which had been treated with larger doses of the male substance were less effective (55). The production of pituitary adenomata by estrogens was prevented by testosterone propionate. The gonadotropic potency of rat pituitaries is maintained throughout pregnancy; the male pituitary is less susceptible than the female pituitary to inhibition by estrogens; testosterone plus x-substance and thyroxin are also inhibitory. Thyroid extract and placental tissue stimulate the pituitary (56). The successful culture *in vitro* of pituitary tissue, of placental tissue, of ova, and of bone marrow opens new avenues of research (57 to 67).

#### GROWTH

Hypophysectomy impairs the trophic state and the functions of somatic cells, and of cells in the organs of internal secretion. Cell replacement, however, is bound to proceed as long as the animal survives (68). Sporadic mitoses have occurred even in the absence of the hypophysis or of any other known endocrine gland; this indicates that mitogenesis is independent of the direct influence of hormones, and is related instead to the trophic condition of the cells. The pituitary hormones, either directly or by their effects on other glands, have a trophic effect upon many cells, and optimal growth of the body may require the combined action of various hormones.

Formerly the effect of the pituitary on growth was tested in

"plateaued" or hypophysectomized animals (69 to 73). Certain effects upon the skeleton were described (74 to 76). The assumption that there is a growth hormone was supported by the fact that the potency of extracts to increase weight was unchanged by purification, even though their lacto-, thyreo-, and gonadotropic principles were largely removed. The importance of these last-mentioned hormones for the growth of dwarf mice or pigeons does not disprove the existence of a growth hormone. Recent work has indicated that the effect of growth hormone is localized in the proliferating zone of cartilage, and has shown the existence of a chondrotrophic effect in certain highly purified pituitary extracts (77 to 80). The evidence for this is supplied by x-ray pictures of the tails of young hypophysectomized rats, by growth in tail-length, and by the histology of epiphyseal cartilage. Growth hormone should be administered in cases in which the pathology reveals a deficient proliferation of cartilage without disturbance of ossification.

*Chemistry of the growth hormone.*—Growth hormone is thermolabile between pH 5 and pH 8. Purification has been attempted by precipitation with ammonium sulphate, by isoelectric precipitation at pH 4.5, and by dialysis. Highly purified preparations (81), active in doses of 10  $\mu$ g. and 3  $\mu$ g., were obtained by adsorption on active charcoal (Norite), elution by liquid phenol, isoelectric precipitation (glass electrode), and final precipitation in a mixture of alcohol and ether. The biuret, ninhydrin, Pauli, and Sakaguchi reactions were positive. The elementary composition was found to be: C, 49.76 per cent; H, 7.24 per cent; N, 14.27 per cent; ash, 2.4 per cent; and S, 1.47 per cent (in the form of dithio groups). Residual nitrogen amounted to 1.28 per cent. A second isoelectric point was found at a pH of 8. The purest product was labile in heat, acid, and alkali, and was destroyed by pepsin and trypsin. It charred at 220° to 230° C., and had a specific rotation of  $-120^\circ$  in .01 *N* alkali. Maximum absorption was observed at 2830 Å and minimum absorption at 2680 Å. The active agent was dialysable and ultrafiltrable at pH 10 through collodion with a porosity of 30 m $\mu$ . Daily injections of 70 units for a period of eleven months resulted in a 60 per cent growth excess in female rats, and signs of incipient gigantism were noted (82).

#### THYREOTROPIC AGENT

The thyroid was enlarged and histological stimulation could be detected in one-day-old baby chicks after injection of graded doses.

of thyreotropic extracts (84). The thyroid of the English sparrow responded to sheep-pituitary extract without refractoriness even after months of treatment (85). A new technique of hypophysectomy in mice is described which is simpler and more reliable than any of the procedures in common use (86). A constant reaction of the thyroid glands of these mice to pituitary extracts opens new possibilities for assay of thyreotropic extracts (87). The cytology of the thyroid shows characteristic features (88), but the conditions and the criteria of assay influence the result; this is illustrated by augmentation or inhibition of the effect by iodine (potassium iodide) (89) and from effects upon the basal metabolic rate and carbohydrate metabolism (90). The hypothalamus also influences the metabolic rate.

A factor thermostable at pH 11 is claimed to be responsible for the metabolic effects of melanotropic, ketogenic, and lactotropic extracts (91). Labile to trypsin, but not to pepsin, this factor is adsorbed by charcoal. It occurs in highest concentration in the pars intermedia; its relations to the ketogenic factor, and to insulin and epinephrine are being studied as well as its behaviour in electrodialysis (92, 93). At least one metabolic effect of pituitary extracts seems to be independent of the thyroid.

*Chemistry.*—A thyreotropic extract of 0.1 mg. per unit was prepared by precipitation with sulphosalicylic acid and sodium tungstate, and removal of the tungstic acid by barium hydroxide and barium chloride; barium was removed by sodium sulfate; final precipitation was effected with benzoic acid, acetone, and ether (94). The flavianic acid compound of thyreotropic hormone from beef pituitary was effective even after a thirty-day period of treatment; the "free" preparation when used for thirty days produced refractoriness (95). A portion with thyreotropic activity could be recovered from the filtrate left after isoelectric precipitation (pH 4.0 to 4.2). The flavianic acid precipitate is redissolved in an alkaline medium when it is used (84). An air-driven ultracentrifuge, which *in vacuo* rotated with a speed of 51000 r.p.m and developed a centrifugal power from 100000 to 200000 times gravity, was used to fractionate a flavianic acid extract of the pituitary, and also flavianic acid compounds of gonadotropic serum and urinary extracts. A preferential sedimentation of the thyreotropic fraction was demonstrated by the baby chick test (96, 97). A reliable and definite separation of the gonadotropic and thyreotropic fractions of the pituitary has not yet been achieved. After being centrifuged at pH 5 the filtrate contained an electrodialysable

thyrotropic fraction, which at 220 volts and a low amperage migrated to the cathode and resisted boiling at pH 3; extracts with gonadotropic activity resisted boiling at pH 7 or 8 (68).

#### CORTICOTROPIC AGENT

The adrenals of hypophysectomized or infantile rats are enlarged, and the sudanophobic zone of the cortex, which occurs in the absence of the pituitary, is reduced by corticotropic treatment. In hypophysectomized mice (98) treatment with pituitary extracts caused large adrenals with nuclear and cortical cell hypertrophy. The Golgi net of cortical cells is characteristically affected by hypophysectomy and replacement therapy (99). Thyroxin and exertion cause cortical hypertrophy; high potassium and low sodium diets have no effect (100). Confirmatory studies were made of the loss of basophils in the pituitary and decrease of blood iodine during fat feeding and corticotropic treatment; of a lack of compensatory hypertrophy of one adrenal after complete hypophysectomy, and of marked hypertrophy of one adrenal after incomplete hypophysectomy (basophilism in the residual tissue) (101 to 104). An excess of "cortin" and sodium and a decrease in potassium were found in the blood of patients with the Cushing syndrome (105). In view of the demonstration of sex hormones in normal and pathologic adrenals, it is important that corticotropic extracts not only enlarge adrenals in infantile or hypophysectomized rats of both sexes, but produce effects after castration of such animals, which are ordinarily attributed to sex hormones, *viz.*, estrus and enlargement of the male organs (106, 107).

*Chemistry.*—Corticotropic activity is present in the precipitate obtained at pH 5 from extracts of cattle, sheep, and pig pituitaries. The yield is considerably increased by pretreatment at pH 10 for two hours at room temperature. The slight amount of corticotropic substance that is present in the filtrate at pH 5 is greatly exceeded by that in the precipitate obtained at pH 5 from which it can be extracted by dilute alcohol or acetone at pH 2.5. The adrenotropic factor is resistant to boiling at pH 3 and pH 8, perhaps more so than prolactin (68). The separation from prolactin, so as to save both, still seems difficult, but successful attempts by fractional precipitation at pH 5.5 and 6.5 have been reported (107, 108).



## GONADOTROPIC AGENT

The multiplicity of gonadotropic factors, their effects, and their origin are still open problems. The type, duration, and criteria of an experiment seem often to decide the response, rather than the alleged "fractionation" of extracts. The rate of absorption and the phenomenon of refractoriness complicate the interpretation of results. An exact knowledge of ovarian ontogeny seems essential. The central position of the egg was emphasized in a recent study. The growing egg acts as the mitogenetic center of the granulosa and theca interna of the follicle. The theca cells become interstitial after follicle atresia, and the interstitial tissue contributes cells toward the formation of corpora lutea. The regression of a follicle may occur at any stage of its development, and is initiated by the extrusion of the polar body of the egg, which at this time loses its function as a mitogenetic center (109 to 111). The trophic condition of interstitial cells is maintained by the hypophysis. The ovotrophic factor is extra-pituitary. In hypophysectomized rabbits estrogens as well as gonadotropic extracts can stimulate the ovary to luteinisation and maintenance of corpora of pregnancy (112). Testicle stimulation by testosterone propionate after hypophysectomy was confirmed (113).

*Methods of assay.*—Opening of the vagina, estrus (114), uterine growth of immature mice (115) and rats (116), and combinations of these criteria have been recommended (117). Sera of pregnant mares produces follicle development and luteinisation (118); follicle maturation in the anestrus ferret, uterine growth of immature guinea pigs and rabbits, and ovulation in the estrous rabbit are associated with gonadotropic effects (119, 120). Mare serum enlarges the testes of chicks (121).

*Biology.*—Gonadotropic extracts of the pituitary induce maturation of the ovaries in hereditary dwarf mice (122). In the vixen ovary as in that of the bitch, the follicles lutenise peripherally before ovulation; extracts from the urine of pregnant animals stimulate estrus, but their effects upon ovulation and fertility are rather doubtful (123). Pituitary extracts are effective in pigeons (124) and in fishes (125). A gonadotropic agent was found in dog urine after injection of extracts from urine of pregnant animals, but not after injection of mare serum (126). The latter produces "superfecundation" in immature rats (127). Pregnancy urine favors estrus in mares and may be used to advantage in animal husbandry (128). The phos-

phatide fraction of hens' blood is increased during laying or after injection of mare serum or pituitary extract; pregnancy urine has no such effect (129). Certain pituitary extracts (the "luteinizing" fraction) promote the disappearance of corpora lutea (130). The possibility is suggested that the "luteinizing" factor may be an inert fraction which augments certain gonadotropic effects, just as casein and zinc sulfate do, by modifying the rate of absorption (131). The rapid effect of serum from pregnant mares upon the ovaries of women was ascertained at operation a few hours after injection (132). Male urine contains a gonadotropic agent similar to that found in the urine of climacterics (133). In rats, inhibition of the pituitary by estrone can be corrected by simultaneous treatment with gonadotropic extracts (134). In rabbits, the "follicle-stimulating" fraction is held responsible for the bleeding during proliferation of the uterine mucosa, after injection of pregnancy urine and pregnant mare serum (135). The effects of human pituitary extracts and of pregnancy urine are not augmented by zinc sulfate (136). Effects of pregnant mare and of horse pituitaries are increased by 0.02 to 0.07 per cent merthiolate (137). Injection of pregnancy urine cures and prevents the "prostate hypertrophy" induced in dogs by estrone treatment (138).

*Chemistry.*—Eighty per cent saturation of extracts of sheep pituitaries with ammonium sulfate and precipitation with flavianic acid are claimed to yield a specific "interstitial" factor for rat ovaries and testicles (139). One hundred thirty-fold purification and 560-fold lowering of the nitrogen content were obtained by fractional precipitation of inert material in mare serum extract, using 50 per cent alcohol or acetone at pH 7 to 7.5, and 70 per cent alcohol at pH 6 (140). Carbohydrates have been found in gonadotropic extracts, 6 per cent in extracts from the hypophysis, and 19 per cent in extracts from urine of pregnant women. It was postulated that mannose and galactose were present (orcin-sulfuric acid reaction), and the possible relation of the active substance to glycoproteins and hexosamines was discussed (141, 142).

#### REFRACTORINESS

Alleged "anti-hormones" are generated by long treatment with the hormone, which they completely "neutralize." The immunological conception is supported by examples of passive immunization by the blood of pretreated donors. Refractory conditions may, however, arise from hormone antagonism. In this case treatment with one hormone causes

the tissue to become unable to respond to another hormone. Refractoriness may also arise from unfavorable circumstances for the action of a hormone. Immunological, hormonal, and circumstantial refractoriness and their inverse, potentiation, play a considerable part in the physiology of the pituitary hormones and the so-called pituitary-like hormones.

*Immunologic refractoriness.*—Several studies are reported on the specificity of antisera (143 to 148). Acetone and ammonium sulfate precipitation, and Seitz and Buchner filtration were used for purification and conservation of sera (149 to 151). Heat, digestive enzymes, and 0.1 *N* sodium hydroxide destroyed antisera, but dilute acid did it no harm. In an incubated mixture, antigen and antiserum neutralized each other *in vitro*. The components of this mixture could be separated and activated again by acid or alkali (152). Rabbits, when pretreated with beef pituitary extract, yielded sera which "selectively" neutralized the "luteogenic" agent. Augmenting and inhibiting sera of sheep and goats were obtained by pituitary pretreatment (153). Pretreatment with muscle extract made rabbit serum anti-thyreotropic (154). The active agent accumulated in the pseudoglobulin and euglobulin fractions of the serum (155). Studies of the immunochemistry of tyrosine derivatives may increase our understanding of serum refractoriness (156 to 158). Normal human blood is anti-thyreotropic in guinea pigs, while that of thyroid patients is inert, irrespective of their disease (159). The weights of rabbit ovaries increased during a nineteen-day treatment with pregnant mare serum, but decreased after that in spite of continued treatment. Gonadotropic agent was present in the blood during nine days of this treatment, but disappeared as the neutralizing power of the blood increased (160).

*Hormone refractoriness or synergism.*—Sheep-pituitary extract is synergistic with pregnant mare serum (161). Continued treatment with pig pituitary changes the pituitary of rats to the type found in castrated animals, along with changes of the ovaries similar to those caused by pituitary deficiency (162). Thyroxin is antigonadotropic (163). Intraperitoneal injection of beef pituitary prevents the gonadotropic (luteogenic) effect of many other gonadotropic extracts. Intraperitoneal pretreatment gives refractoriness during at least eight days. This has been interpreted as a local "peracute refractoriness," rather than as evidence of an "antagonistic" hormone of the pituitary (164).

*Circumstantial refractoriness or synergism.*—Blockade of the re-

ticuloendothelial system or splenectomy increases gonadotropic effects (165, 166). Pituitary extract prepared by sodium carbonate extraction is less antigenic (167, 95).

#### PREGNANCY, LACTATION, AND THE PITUITARY

The extract of the pituitary of pregnant cows and pigs is claimed to give mammary gland proliferation in castrated rabbits (168). Progesterone in 2 mg. doses was found inert (169). The placenta, without the fetus, is able to maintain pregnancy changes in the rat (170). Lactation does not occur until term in pregnant rats, when the fetus develops in the amnion sack after cutting open the wall of the uterus (171, 172). In female goats estradiolbenzoate induces proliferation of the mammary gland (173). The same is true of monkeys (174). Proliferation of the mammary gland and lactation are definitely different endocrine processes. A recent short review (175) compiles some facts and views about lactation. The existence of more than one lactation hormone in the pituitary is possible (176). Fresh saline extract of the pituitary proved more effective than prolactin for inducing increased lactation in the cow (177). Hypophysectomy and treatment with steroids inhibit lactation. Other endocrine organs also affect lactation (178). Prolactin is important in the development of the maternal instinct (179).

The separation of prolactin from other active agents of the pituitary was attempted by means of precipitation with copper or zinc hydroxides at pH 7. In the presence of copper little prolactin is soluble, as compared with other active material; 0.2  $\mu$ g. per pigeon was found to be the lowest effective dose (180). By the method of du Vigneaud, Jensen & Wintersteiner a further purification of Lyons's "mammotropin" was accomplished. A crystalline product of protein nature resulted, of which the lowest effective dose was 0.1 to 0.2  $\mu$ g. when it was applied locally to the crop. Analysis gave C, 51.11 per cent; H, 6.76 per cent; N, 14.38 per cent; and S, 1.77 per cent. It gave a positive tryptophane reaction, and when tested with x-rays gave a diffraction pattern with two strong lines at 88.5 Å and 43 Å (181).

#### METABOLISM AND THE PITUITARY

*General and nitrogen metabolism.*—Tolerance to parenterally administered gelatin has been recommended to test the state of nitrogen

metabolism in pituitary deficiency (182). Blood protein and non-protein nitrogen concentration were investigated in this connection (183, 184). The hypophysectomized rat has a metabolic rate approximately 40 per cent lower than that of the normal animal, as determined by means of oxygen consumption, but the R.Q. is high. Carbon dioxide production is less impaired by lack of the pituitary, but this is raised as in normal rats by oral administration of glycine (185). In hypophysectomized rats the total water (67.8 per cent) increases slightly more than protein (20.2 per cent) after treatment with "growth hormone." The increase of total water is 33 to 35 per cent, and of total protein 19 to 48 per cent. A low "water capacity" of the cells seems to be a basic change after hypophysectomy, while fat metabolism proceeds as usual, but no fatty infiltration of the liver occurs after phosphorus poisoning (186).

*Carbohydrates.*—A recent review surveys the relation of the pituitary to carbohydrate metabolism. The pituitary increases the anabolism of carbohydrate, and perhaps also promotes its production from other metabolites. The effect does not depend directly on the participation of the adrenals. The glycogen stores of muscle are protected by the pituitary, and an assay could be based on the requirement that pituitary extracts should restore muscle glycogen to the level of 0.45 per cent after hypophysectomy (187). The importance of blood glucose for the stimulation of a glycotropic secretion of the pituitary (188), the low resistance of rabbits to insulin after ligation of the hepatic vessels, and the resulting blockade of the glycogen reserves (189), and the discovery of the presence of glycotropic factor when the level of blood sugar is lower than 60 mg. per cent (190) are recent contributions to this subject (191, 192).

An effect of pituitary extracts upon the islets of Langerhans seems to be demonstrable (193, 194). Glucose resorption and insulin resistance are decreased in hypophysectomized rats (195). Thyroxin restores resorption of glucose, but does not replace the hypophysis in carbohydrate metabolism (196). Pituitary extracts produce a diabetic condition in dogs and in rats (13). The equivalent of 0.004 gm. of fresh pituitary (extracted at pH 9 to 10 with 60 per cent alcohol) increased the level of blood sugar in guinea pigs by 50 per cent (197). The equilibrium between liver and muscle glycogen is shifted towards the muscle glycogen after treatment with pituitary extracts (198). The gradual action of the glycotropic or diabetogenic factor of pituitary extract differentiates it from the hyperglycemic factor, which

acts immediately. The diabetogenic factor is not identical with prolactin. At pH 5.5 prolactin precipitates, and the filtrate left may be further purified by separating the pseudoglobulin fraction; the diabetogenic substance in this is heat resistant at pH 10 (199). Separate diabetogenic and ketogenic factors have been postulated (200, 68). In pigeons, prolactin seems to have diabetogenic potency (201).

Since the study of the ketogenic factor is less advanced, we mention here only five papers at random by way of reference (202 to 206).

### THE COLCHICINE TECHNIQUE

Subcutaneous injection in water or oily solution of 0.5 to 4  $\mu$ g. colchicine per gram of body weight causes mitoses all over the body to be arrested in metaphase within six to fifteen hours after injection. Where mitotic activity depends in some way on hormone effects, evidence has been gained that cell division depends on hormone treatment rather than on colchicine. It seems more probable, therefore, that mitosis is arrested but not induced by colchicine. The interest in using this technical aid for endocrine investigation has steadily been growing and, although it is desirable that in parallel experiments mitotic activity without colchicine should be used as a control, colchicine opens up many new possibilities. The bibliography of this technique is presented (207 to 222).

### INSULIN

The method used for standardizing insulin, which is based on years of experience, has been described in detail (223). Intravenous injection of insulin shows no advantage in the standardization (224). The use of the polarograph has been studied, with the intention of finding a physical method which will measure satisfactorily the value of a preparation. Various commercial insulins with the same biological activity showed different heights in the curve when examined polarographically. This difference was also noticed with some crystalline preparations; consequently the method is not generally applicable (225 to 227).

It appears that the hypoglycemic property of insulin is associated

with certain dithio groupings which are present, in part at least, as combined cystine, and also with certain free amino groups which are most likely present as cystine (228). It has been shown that the sulfur in insulin can be entirely accounted for as cystine. Not more than traces of other sulfur compounds can be present (229). According to the experimental conditions, two varieties of acetylinsulin have been produced by acetylation of insulin with ketene; only the free amino groups are acetylated by short treatment, but after a longer time the hydroxyl groups of the tyrosine in insulin are also acetylated. The products from the first reaction are easily soluble in water; their activity is unchanged. The tyrosine acetyl derivatives are insoluble in water, and are only slightly active physiologically (230).

It has long been known that insulin which has been boiled with hydrochloric acid is inactive when it is injected. If this inactive insulin is treated for a short time with alkali, however, it recovers its activity. The acid-treated product is active when it is given orally in large doses to mice; it produces convulsions and lowers the blood-sugar content. This action can be intensified by iron salts (231). When hydrochloric acid is added at low temperatures to aqueous solutions of insulin, a precipitate is formed from both insulin and zinc-insulin, which has high activity but differs from the starting material (232). A new kind of insulin system which has protracted action has been established with bis-2-methyl-4-aminoquinolyl-6-carbamide hydrochloride (Surfen); it is the result of numerous experiences with the prolonged effect of protamine-zinc-insulin (233).

Totally pancreatectomized dogs have been kept alive for four to five years with insulin and a diet high in calories and rich in protein and vitamins; the required daily dose of insulin amounted to 16 units (234). In an accurate investigation, the minimum dose of insulin for pancreatectomized dogs was found to be 0.035 to 0.005 units per hour per kilo body weight (235). The quantitative relationship between glucose and insulin in dogs was investigated by means of a special experimental arrangement for insuring continuous intravenous infusion (236). Pancreatectomized diabetic dogs, which had been well standardized with insulin, showed a disturbance of metabolism after five to seven weeks on a diet practically free from vitamin B, which was characterized by a rise in the blood sugar, glycosuria, and a lack of response to insulin; later almost complete insulin resistance occurred. In spite of excessive doses of insulin, hypoglycemic phenomena did not appear (237).

## THE THYROID

Contrary to results with diiodotyrosine and thyroxin, thyroglobulin increased the oxygen consumption of the livers of rats and guinea pigs. Diiodotyrosine caused no alteration in the oxygen consumption of guinea-pig liver, but with rat liver a diminution always resulted. From these experiments it is inferred that the thyroid hormone is not thyroxin, but either thyroglobulin itself or an intrinsic part thereof; thyroglobulin is stored in an inactive form by the colloid substance of the thyroid gland, and subsequently liberated (245). From investigations of the action of epinephrine on blood pressure it is suggested that two substances are present in the thyroid gland which act on the autonomic nervous system; one of them sensitizes the sympathetic system, is found in the epithelial cells, and is insoluble in alcohol and acid-alcohol; the other sensitizes the parasympathetic system, is present in the colloid substance, and is soluble in alcohol and acid-alcohol (246). A relationship exists between the nature of the ash of the thyroid gland and its physiological activity (247).

Artificial iodized protein derivatives, which have an action similar to thyroid extracts, have so far been obtained only from unmodified natural proteins like casein, serum albumin and globulin, egg albumin, etc., and not from degraded proteins such as peptones. The nature of the iodination, as well as the constitution of the proteins, plays an important rôle. A direct connection between physiological activity and the thyroxin-iodine content cannot be detected in iodoproteins (248). The thyroid gland probably contains more active iodine compounds than the synthetic thyroxin preparation or, perhaps, iodine-free substances which increase the effect of this drug (249).

The preparation and properties of thyroxin-O-methyl ether, O-methyl-N-methyl-diiodothyroxin, and O-methyl-N-methyl-diiodotyrosine have been described and their biological effects examined. O-methyl-N-methyl-diiodotyrosine is inert. The action of thyroxin-O-methyl ether is greater than the action of thyroxin on body weight, energy metabolism, and the carbohydrate metabolism of the liver. The reactivity of the thyroid gland towards thyreotropic hormones is depressed by these compounds (250).

Hyperthyroid rats fed with highly unsaturated fatty acids showed antithyroid reactions proportional to the number of double bonds in the fatty acids fed. Linolenic acid, however, with three double bonds was rather less active than linoleic acid with two; this was explained



by its poor compatibility and its defective resorption. The ability of the liver to build up glycogen from sugar, which is impaired by hyperthyroidism, is not improved in male rats by the feeding of unsaturated fatty acids as it is in female rats (251).

Dogs which have been treated with "antithyreoidin Moebius" or with thyroid gland substance, eliminate a substance in their urine which affects adversely the metabolism-stimulating action of thyroxin. A petroleum ether fraction may be obtained from the urine of men, which affects synergistically the action of thyroxin on metabolism. At the same time, one portion is obtained from the ether-soluble fraction, which on injection inhibits the action of the thyroid gland substance in increasing the metabolic rate (252).

Glycine, which has scarcely any effect on the basal metabolic rate also shows an antithyroid action (253). Similarly, rabbit serum may be shown to contain an antithyroid substance which is formed in the pancreas (254). In men, vitamin C has no antithyroid properties (255).

The effects on the organs of eighty rats chronically poisoned with acetonitrile were exhaustively investigated (256). Resistance towards acetonitrile could be increased very considerably by thyroxin, as is the case with thyreotropic hormone in mice (257).

The elimination of iodine in the urine is subject to fluctuations arising from the iodine supply in the food and geographical location. The urine of patients with hyperfunction of the thyroid gland contains almost three to four times more iodine than that from patients with healthy thyroid glands. Patients with toxic adenoma of the thyroid gland eliminate more iodine than those suffering from Basedow's disease. Estimation of the iodine content of the blood and the urine can be of value for differential diagnosis (258).

The intensity of the action of insulin, and its duration, are increased in Basedow's disease and in myxedema. In the former this phenomenon is due to a lack of glycogen in the liver; in myxedema large amounts of glycogen are available, but the mobilizing action of epinephrine is lacking. In thyroidectomized rabbits an increasing supersensitivity to insulin develops in the course of a few weeks (259).

Opinions are very divided on anomalies in the blood picture in cases of hyperfunction of the thyroid gland; when alterations occur they are very small. In cases of thyroid hypofunction there is a tendency to anemia which may be both hyper- and hypochromic. It has been observed that in the absence of the thyroid gland, the regenerat-

ing powers of the haemotopoietic organs are to a large extent lacking (272, 273). Saponin-collargol anemia is not cured by liver extract in thyroidectomized rabbits, except when a thyroid fraction is injected; the active agent is not identical with thyroxin, since it does not increase the basal metabolic rate (274). In rats the reticulocytes practically disappear, one to two weeks after hypophysectomy. Thyroidectomized rats possess a normal number of reticulocytes. If hypophysectomized rats are given sufficient thyreotropic hormone to show a marked activity on the thyroid gland, the reticulocytes do not return. Atrophy of the thyroid gland cannot be the cause of their disappearance after hypophysectomy (275). In experimental hyperthyroidism in rabbits, guinea pigs, and cats, and also in Basedow cases there is definite atrophy of the bones (276). There is also an increased occurrence of young pseudoeosinophilic cells, which indicates an increased function of the bone marrow (277).

Activation of the thyroid immediately after ovariectomy, and the inhibition of its function by large doses of estrogenic substances point to a close connection between the ovary and the thyroid gland (278). This decrease in thyroid function results from suppression by estrogenic substances of the production of thyreotropic hormones in the anterior lobe of the pituitary gland (279, 280). In rats, the whole genital apparatus must be uninjured for the ovaries to act on the thyroid gland. The action of the ovary is connected with that of the estrogenic substance, which attains active thyroid gland properties only after passage through the uterus (280).

In rabbits which are thyroidectomized during pregnancy the typical rise in basal metabolism does not occur. Removal of the thyroid gland has no effect on the ability to conceive, the course of pregnancy, the vitality of the offspring, or the maternal care (281).

Corpus luteum hormone alone has no action on the thyroid gland (282), nor can progesterone check the action of estrone on the thyroid gland (283).

## ADRENAL CORTEX

A recent review deals with several aspects of the physiology of the adrenal cortex (286).

### ASSAY AND BIOLOGY

It is important to spare the splanchnic nerves during adrenalectomy in dogs (287). Sodium excretion is inhibited in normal dogs

by large doses of cortical extracts (288, 289), but in man, a curious immunologic refractoriness is said to develop against this effect, and also against the metabolism-stimulating effect (290). It is claimed that the kidney contributes to the increased potassium level in the blood after adrenalectomy, by failure to eliminate potassium (291). Adrenalectomized dogs have a "negative" water balance (292). In cats with diabetes insipidus, adrenalectomy affects water intake rather than excretion (293), and the potassium content of the blood is increased relatively more than the sodium content is decreased (294). A contributing factor to the disturbed water balance is probably the decrease of phospholipids of the plasma, as compared with those of the cells (295). Cortin is diuretic (perhaps primarily thirst-promoting), but conserves salt, while posterior pituitary extracts promote excretion of salt with little water. In the opossum, anterior lobe extirpation is diuretic only until the adrenal atrophy has reached an advanced state (296). The metabolism of adrenalectomized rats is less stimulated by exposure to cold than that of normal rats (297).

In adrenalectomized rats the sodium chloride and sodium bicarbonate levels are low, while the potassium and residual nitrogen levels are higher than in normal rats; the potassium in muscle is increased, and intracellular water is inversely proportional to external sodium concentration (298). A diet containing more sodium and less potassium greatly improves the muscular power of rats in the tread mill and "swimming test" and prolongs life (299). In rats, cortin improves the performance of fatigued muscle only (300). After adrenalectomy, phosphorus intoxication did not result in fatty liver (301). Lactoflavin and cortin together prolong the life of adrenalectomized rats; lactoflavin alone does not do so (302). Glycine resorption is disturbed after adrenalectomy of rats; the importance of cortical substances for hydration is pointed out as one of the possible causes of this phenomenon (303). The adrenals are unimportant in bringing about mitosis in the rat intestine (221). Chickens were observed to die within a few hours after cauterization of the adrenals, unless they were treated with cortical extracts and drinking water which contained 1.6 per cent sodium chloride (304).

Transplantation of adrenals succeeded best when a young donor was selected, closely related genetically to the host. Regeneration of implanted cortical tissue after removal of the host's adrenals proceeded from the capsule (305 to 307).

## SEXUALITY AND ADRENALS

The X-zone, especially in the cortex of mice, is the source of substances which affect sexual phenomena, but which are unimportant for the maintenance of life (308). Castration has marked effects upon the size and histology of the adrenals (309). Silver nitrate impregnation is very suitable to show the X-zone (310). Substances other than cortin are thought responsible for certain effects of adrenal extracts upon sexual phenomena and the sex organs (311 to 313).

## CHEMISTRY

The chemistry of the substances isolated from the suprarenal cortex has been reviewed (314). Up to the present about twenty different closely related sterol derivatives have been prepared from the suprarenal cortex. The last of these substances is called substance S in the alphabetical nomenclature used by the Swiss workers. It is isomeric with corticosterone (315). The greatest interest is attached to substance Q, because it has been shown that this is identical with desoxycorticosterone. This proves that this active substance, which so far has been made only synthetically, is also a constituent of the suprarenal cortex (316). Substance M is half as active as corticosterone, while substance E is only one-sixth as active (317). The formulae for substances C, D, E, F, and G have been confirmed by American workers (318, 319). Substances L and N of the Swiss workers have proved to be identical with the previously (320) isolated compounds G and H (321) of the American investigators (322, 323).

The carbon skeleton of corticosterone has been worked out by systematic degradation reactions and by conversion of the final product into allopregnane (324).

The biological activity of a number of esters of desoxycorticosterone has been examined, starting from studies of the effects of prolonged use of esters of the sex hormones. With esters of the lower fatty acids the potency of the effect, but not its duration, was increased; the palmitate ester prolonged the effect to more than three weeks (325).

## THE OVARY

Twenty-five per cent of the estrogenic activity of the human placenta is found to be due to ketones now identified as estrone (326).

Likewise, the ketonic estrogen which is responsible for 15 per cent of the activity of the sow ovary, is probably estrone (327). Male urine also owes half its estrogenic activity to estrone (328). The remaining estrogenic activity is caused by non-ketones.

Hydrogenation of equilin affords chemical proof that the double bond is situated between carbon atoms seven and eight (329). The hydrogenation product is iso-estradiol (isomerism on the eighth carbon atom) which gives iso-estrone on oxidation. The estrogenic activity of the iso compounds is only one-third as great as that of estrone compounds on the average. The estrogenic activity is practically destroyed by the introduction of a hydroxyl group in ring II of equilin. Hydrogenation of equilenin produces both 3-epimeric 17-trans-hexahydroequilenins, which may be obtained also by hydrogenation of  $\alpha$ -dihydroequilenin. Hydrogenation of  $\beta$ -dihydroequilenin yields the 3-epimeric 17-cis-hexahydroequilenins (330 to 332). The estrogenic activity of these hexahydroequilenins is extremely small, which is all the more remarkable, because the supposed estrone isomer (ring B, aromatic) has a very high activity (333). The colorimetric method of determining estrogenic substances has been improved by using  $\beta$ -naphthol sulfonic acid (334).

Volatile substances with estrogenic activity are found in certain kinds of honey (335). The estrogenic activities of fennel and aniseed oils may possibly be ascribed to the anethole present in them (336).

Sterol derivatives containing a nitrogen atom in the 4-position have no biological activity (337).

Investigations on the degradation and synthesis of equol from the phenolic fraction of the urine of pregnant mares, with a view to determining its structure, have appeared, and two possible structures for equol have been discussed (338).

The progesterone-like action of suprarenal extracts has been traced back to progesterone itself, for progesterone and 3,20-allopregnanolone have been isolated from ox adrenals (339, 340). Ethinylestradiol and ethinyltestosterone are interesting compounds which have been prepared from estrone and dehydroandrosterone (341, 342). When it is administered orally, ethinylestradiol is greatly superior to estradiol; it inhibits the imbedment of the ovum in rabbits and interrupts pregnancy (343). Ethinyltestosterone shows an action similar to progesterone.

Catalytic hydrogenation of pregnanedione affords *n*-pregnane-3-ol-20-one and *epi*pregnaneolene (which is isomeric on the third car-

bon atom). Both compounds are inactive on capon combs (344). Two triols have been isolated from the sterol fraction of the urine of pregnant mares. Pregnanetriol A has hydroxyl groups on carbon atoms 3, 11, and 20; its structure probably differs from that of pregnane by asymmetry at the ninth carbon atom. The name urane, which is inappropriate and liable to lead to confusion, has been proposed for the new hydrocarbon, and urantriol for the triol (345, 346). Pregnanetriol B agrees in properties with a previously isolated triol (347, 348).

*Epi-allopregnaneolone*, which was discovered in the urine of pregnant women, has been regarded as the male active substance in this urine (349). When it is prepared synthetically, however, it is without action in either the capon test or the vesicular gland test (350). Various steroids have been isolated from the urine of a woman with a suprarenal tumor (351).

So far, diethylstilbestrol (352), which is extremely active in producing estrus, acts similarly to estrone or estradiol (353 to 360). The various esters of diethylstilbestrol show a somewhat weaker estrogenic action, but they act over a longer period. Triphenylethylene also acts in a manner similar to the estrogenic hormones (361, 362). Triphenylchloroethylene has a considerably more prolonged action than estradiol benzoate (363). When applied to the comb of a capon together with testosterone, the estrogenic hormone shows an antagonistic action, while diethylstilbestrol does not; therefore, a biological differentiation between these two groups of substances is possible (364).

The detection of male active substances in ovaries by transplantation experiments has been confirmed (365, 366). This androgenic action is weak, and probably is not to be ascribed to testosterone (366). Comb-growth activity can also be detected in extracts of sow ovaries (367). The male active substances eliminated in the urine of women probably originated from the suprarenal glands (368).

From recent investigations it can now be considered as certain that the normal menstruating woman has two peaks in the excretion of estrogens in urine, one at mid-interval, and one shortly before menstruation (369, 370). The peak at mid-interval corresponds with the rupture of the follicle and varies in individual cycles; therefore, it may be concluded that ovulation does not always occur at the same time. Further, clinical examinations have shown that of forty-seven women studied postpartum, 55 per cent of the cycles were anovula-

tory, and the bleeding did not come from a premenstrual endometrium (371, 372). The second peak appears with the development of the corpus luteum. During menstruation the amounts eliminated are extremely small. It is assumed that estradiol is the substance produced primarily in the ovary, and this is converted through estrone into estriol. The last stage is dependent on the amount of progesterone present in the body, so that the elimination of estriol in the luteal phase is relatively high. The ratio of estriol to estrone in the urine changes during pregnancy; the proportion of estriol constantly increases; this may be traced back to an increasing production of progesterone in the placenta. Shortly before birth a sharp fall occurs in the elimination of estriol, and the amount of estrone eliminated increases. However, this does not apply to all cases of pregnancy (373).

In the mare, the largest amounts of estrogenic substances are eliminated between the 168th and the 252nd days of pregnancy; a sharp diminution occurs on the 280th day. There are very large variations, both within the individual and between different individuals (374). From differences in the elimination of male and female hormone in the urine, and in agreement with tests on animals, it is assumed that at the epiphyseal closure the estrogenic hormones play a more important part than the male hormones (375).

After administering large amounts of estrone to rats, only very small amounts were recovered in the urine and feces, and only a small proportion was recovered from the body six hours afterwards. Six hours after administering estrone benzoate, the bulk could be recovered unchanged, and fifty per cent of the estrogenic units were still unabsorbed as long as twenty-four to forty-eight hours after injection. After injection, the benzoate does not circulate through the body as such, but as free hormone; hence its protracted action is caused by delayed absorption and not by the slow hydrolysis of the ester in the body (376). More than ninety per cent of estrone disappears from the blood stream of dogs within a few minutes after intravenous injection. About thirteen per cent is excreted in the urine. It is also excreted in the bile, and only one per cent is found in the liver, spleen, and kidneys (377). In women, three to twelve per cent of estrone given intramuscularly is found in the urine shortly after the injection. The amount eliminated after oral application is somewhat larger (378). Estrone is inactivated after forty-eight hours by the hepatectomized frog; therefore, the liver cannot be the only organ in which

inactivation can occur (379). No increase of estrogenic substance could be detected in the whole bodies of male, female, or castrated rats after irradiation with x-rays (380). The maternal liver and the fetal liver contain more estrogenic substance during pregnancy than the placenta. The fetal adrenals contain as much estrogenic substance as the placenta, or more. The general cholesterol metabolism is a more likely source of the estrogenic substance during pregnancy than is the placenta (381).

The Allen-Doisy test has been found to be the most satisfactory method of standardization, after summarizing experimental results over many years (382, 383). Estrus in guinea pigs cannot be definitely proved by means of vaginal smears, but touching the guinea pigs in a definite manner elicits a characteristic reaction. In exceptional cases estrus occurs without the vagina having opened (384). In guinea pigs estrogen-induced estrus and estrogen-progesterone-induced estrus differ in several respects (385), so that biological testing and assaying of estrogen and progesterone are possible (386). The growth of the nipples of guinea pigs after local application of estrogenic preparations may also be used for assay (387). In certain doses zinc chloride intensifies and prolongs the effect of estrone in castrated rats (388).

The susceptibility of castrated and hypophysectomized rats to estrone decreases periodically during continued treatment. These cycles of susceptibility disappear after adrenalectomy, even though life is maintained by cortical extracts (389). While the removal of the hypophysis results in a decrease in the estrogenic threshold dose, thymectomy has no effect (390).

Emmenin, estriol glucuronide, and estriol have the same activity on castrated and uncastrated animals. Hence these substances are not converted into more active substances by the ovaries of the animals concerned.

The activity of estriol glucuronide is about ten times weaker when given subcutaneously than when administered orally; on the other hand, estriol is about twice as active when given subcutaneously as when applied orally (391). When estrogenic hormones are injected into the uterus, very small doses lead in a short time to hyperplasia of the musculature and proliferation of the mucous membrane (392). The hyperplasia of the uterine musculature after injection of estrogen is accompanied by pronounced hypertrophy of individual muscle fibres and a considerable increase in their number (393). The in-



crease in the weight of the uterus after one injection of estrogen is accompanied by, and almost entirely caused by, an increase in the water content of the uterus. The maximum is reached after six hours. This increase in weight may be used for the standardization of estrogen (394).

The dependence of uterine bleeding on estrogenic hormones and on progesterone has been investigated in detail in castrated monkeys. Considerable glycogen is found in the uterine glands when estrin is given, and very little is released into the lumina of the glands; progesterone produces a release of glycogen (395). Estrone administered in small daily doses is, in a sense, a sex depressant for the intact female rat, since the latter shows a fairly constant low degree of sexual receptivity during the injection period (396).

A toxic effect of estrogens was discovered by prolonged treatment of dogs. The sequelae of poisoning were agranulocytosis, hemorrhage (petechial) in various organs, and progressive anemia with reticulopenia and thrombopenia, leading to death. The state of the bone marrow corresponded to the successive stages of poisoning (409 to 411). Protracted excessive doses of estrogens caused eunuchoid dwarfism in rats (412). In young rats, estrone retards growth and reduces the abdominal circumference; the pituitary and thyroid glands enlarge somewhat (413). Hypophysectomized rats are also poisoned by estrogens (414). A pyometra in the parts of the uterus which are in contact with the cervix develops in rats after large doses of estrogenic substances chronically administered. Transplantation of pieces of the uterus has shown that estrogenic substances do not cause pyometra, but only promote uterine infection from the vagina (415). Several authors agree that malignant epithelial growths cannot be induced in the uterus and vagina by excessive doses of estrogens (416 to 418). The precancerous, and occasionally cancer-like appearance of the epithelium is probably due to simultaneous mechanical stimulations (419). An occurrence of tumors, diagnosed histologically as malignant, was found in rats treated for long periods with estrone in conjunction with various forms of local irritation (420). From accumulated data it appears that mammary tumors of female and male mice are favored by estrogens only in strains with a high incidence of spontaneous cancer. Diethylstilbestrol acts in the same manner, but is a little more progressive (359). Various types of sarcoma and epithelioma were observed in strains with low spontaneous tumor incidence after protracted estrogen treatment and in cases where treat-

ment must have favored tumor development (421). Excessive doses of estrogens produce a pronounced multicentric metaplasia of the uterine epithelium in rats, which can be prevented by the simultaneous application of progesterone (422).

#### CORPUS LUTEUM

The occurrence of colloid droplets, which are met with only in the corpus luteum of pregnancy and never in corpus luteum menstruationis, has again been described. The colloid is not a sign of degeneration, and has nothing whatever to do with the production of progesterone; it is much more likely an expression of a special secretion, the existence of which has not yet been completely established (423).

Removal of the normal ("cyclic") corpus luteum from the ovaries in sheep leads to ovulation and estrus in the days immediately following the operation; the same applies to the corpus luteum of pregnancy. After the removal of the corpora lutea, ovulation and estrus can be produced only occasionally by injection of gonadotropic hormones from pregnancy urine or from the serum from pregnant mares (424). When the corpora lutea are removed from rats by cauterization between the third and seventh day of the apparent pregnancy, estrus occurs again four days later. When the corpora lutea are destroyed, and the placenta and embryo removed from pregnant animals six or seven days after pregnancy, estrus occurs three to seven days later. Destruction of the corpora lutea alone on the fourth or fifth day of pregnancy results in the onset of estrus on the eighth or ninth day. Removal of the corpora lutea on the eleventh to sixteenth day of pregnancy no longer brings about estrus. Some of the animals miscarry, while in others the embryo is partly resorbed (425).

As a rule, pregnancy in rabbits is interrupted forty-eight hours after ovariectomy, but after daily doses of 0.5 to 1.5 mg. of progesterone, embedded egg cells, as well as live embryos near dead ones, can be observed in the uterus (426). Pregnancy in rabbits can be prolonged to forty-five days by the daily application of 2 mg. of progesterone (427). Experiments on the golden hamster showed that injection of progesterone alone failed to maintain pregnancy, but that it could be maintained with simultaneous injections of progesterone and estrone (428).

The importance of estrogenic hormones for the corpus luteum has been established by a new experimental technique. Interruption of the blood supply of the ovaries of rabbits for one to two hours, and

withdrawal of part of their blood, interferes with production of corpora lutea. The follicles are transformed to cysts or blood follicles. Estrone-rich blood of adult female rabbits restores ovulation and the production of corpora lutea, while blood from infantile, male, or castrated rabbits can restore ovulation and the production of corpora lutea only if the donor has been pretreated with large doses of estrone (429). In rabbits, progesterone does not maintain the mucous membrane of the uterus in the pregravid state; in spite of continued treatment, regression of the mucous membrane occurs. This is not the result of the formation of antihormones towards progesterone; this regression is avoided when progesterone is combined with estrone (430, 431). In young castrated female monkeys treatment with both estrone and progesterone produces a better development of the uterus than administration of either substance alone (432).

Inhibition of estrus in guinea pigs by progesterone has been recommended for detecting this substance (433). "Inhibition of estrus" by hormones is an unfortunate expression. The substances known as estrus-inhibiting, inhibit only cornification, and really promote epithelial differentiation from the non-cornifying, stratified type towards the highly differentiated, mucified type (434).

Up to a certain point, testosterone has an action similar to that of progesterone. Nevertheless, its ability to act as a substitute has its limits; doses of testosterone, which induce the typical condition produced by progesterone in the epithelium of the uterus of castrated rabbits, were not able to maintain pregnancy (435). Furthermore, in rabbits and mice, the sensitivity of the uterus to pituitrin was not lowered by androsterone and testosterone (436-439).

The abdominal window method has proved useful for studying changes in the sensitivity of the uterus of rabbits (440).

The effect of testosterone in conjunction with uterine trauma differs qualitatively both from that of progesterone and that of estrogens (441). The effects of the various male hormones in the female organism have been exhaustively studied in rats (442 to 444), mice (444), and rabbits (445). The existence of an ovarian factor in the rat, which inhibits the uterine response to estrogens and operates in a cyclic manner corresponding with the estrus cycle of the animal, has been demonstrated. It is thought that the effect is not due to progestin (446). The response of the uterus of the cat to epinephrine is inhibited under certain circumstances by estrone and progesterone (447).

## EFFECT OF HORMONES ON SEX

From investigations carried out during the past year it is certain that estrogens tend to produce feminine characteristics in the development of gonads in male chicken embryos (448 to 452). Diethylstilbestrol acts in the same way (451). A similar effect with estrogens is noticed with lizards (453, 454) and frogs (455). Antenatal treatment of mammals with estrogens caused abortion regularly because of the very high doses that were required; this abortive action could be avoided by using estradiol dipropionate, which is very slowly resorbed. All the male offspring of a mother treated with 2 to 4 mg. of estradiol dipropionate had visible nipples at birth, and were hypospadiac. The testis lay abnormally high in the abdominal cavity. Abnormalities were also observed in the female young (456).

It is not possible to influence the female sex of chicken embryos by male hormones (457, 458). However, in mammals it is possible to detect "masculinization" of the female by male hormones. This has been shown in guinea pigs and mice (458 to 463). The female young from a mother which had been treated during pregnancy with male hormones, showed externally a male habitus with penisoid clitoris and a scrotum-like development of the space between the anus and the clitoris, besides normal ovaries and more or less completely formed tubes and uterus. The distal part of the vagina was missing completely in animals whose mothers had been given larger doses of male hormones. At the proximal part of the urethra, a pair of glandular formations similar to prostata were found, as well as other glandular organs which were similar histologically to the vesicular glands in men.

## THE TESTIS

The chemistry of the male sex hormone has been completely reviewed recently (464). Androsterone and trans-dehydroandrosterone have been isolated from the urine of normal women, in yields comparable to those obtained from the urine of normal men (465). To some extent the substances which stimulate comb growth are identical in male and female urine. It is probable that the male hormones eliminated in bulls' urine are  $\alpha$ ,  $\beta$ -unsaturated ketones, or substances closely related to them (466). Substances which promote comb growth

can also be extracted from fish testicles (467). In the preparation of the glucosides of androsterone, dehydroandrosterone, and some other steroids, the ability to attach glucose in the allocholane series seemed to run parallel with the ability to add digitonin (468). By condensing hormones with methylacetobromoglucuronate, the corresponding derivatives of the coupled hormone-glucuronic acids have been obtained in crystalline form. These could be converted into the corresponding coupled acids, such as methyl dehydroandrosterone-triacetylglucuronate and  $\alpha$ -estradiol (3)-benzoate (17)-triacetylglucuronate. Both the free coupled acids and their derivatives were almost inactive biologically (469).

When testosterone is treated with aluminum isopropylate, a mixture of *n*- $\Delta^4$ -androstene-3,17-diol and *epi*- $\Delta^4$ -androstene-3,17-diol (isomeric at C<sub>3</sub>) is produced, and both of these possess relatively high comb-growth activity. The occurrence of the double bond in the 4:5 position is significant for the physiological activity; this is shown by a comparison with iso-androstandiol and *n*- $\Delta^5$ -androstenediol. This difference is apparent only in its action on comb growth, and not in its action on the seminal vesicle (470). However, this question is not yet finally settled, because both  $\Delta^5$ -androstenediol and  $\Delta^4$ -androstenediol have low comb-growth activities when compared with testosterone (471).

When the same amount of androsterone was administered to practically identical sets of birds (in Holland), the growth of the comb had a maximum value in autumn and winter, and reached a minimum in summer. Whether the phenomenon is due to longer illumination in summer, is not yet clear (472).

The effectiveness of crystalline gonadal hormones, administered by subcutaneous implantation of solid tablets of the pure substances, has been investigated further. The technique is particularly useful when a steady effect is required over a long period (473 to 476).

The doses of male hormones smeared on the comb of the capon necessary to produce the same comb-growth activity, differ very slightly from one another, although this is not true when the male substances are injected (477). The use of comb weight of cockerels for measuring growth activity, which has been recommended for some time, has proved thoroughly reliable (478, 479). The combs of cockerels also grow when they are smeared with male hormones (480), but only certain breeds of fowl are suitable. However, when one compares the action of the various male hormones on the combs of

cockerels with the corresponding action on the combs of capons, differences are observed for which there is not yet a satisfactory explanation (481).

Also much smaller doses of male hormones are required to stimulate growth of the seminal vesicles when applied locally to this organ than are necessary in subcutaneous injection (482). In oral application, methyltestosterone clearly proves itself superior to all other male hormones (483).

For various reasons rats have been found to be more satisfactory than mice as test animals for the male hormones (484). Nevertheless, the ejaculation test with mice has been recommended for the biological evaluation of male sex hormones (485). The reactions of the most varied hormones can be readily followed in the prostate and seminal vesicles of rabbits after transplantation into the anterior chamber of the eye (486 to 488).

The optimum action, particularly on growth of the seminal vesicle, is obtained with unpurified testis extracts containing both the comb-growth substances and the X-substance fraction (489). Without exception, the pure-comb growth substances are only slightly active on the growth of the seminal vesicle; peculiarly, the action of those substances which possess a hydroxyl group in the 17-position in the steroid skeleton, is intensified by the X-substance fraction. Incidentally, the purification of the X-substance fraction has been carried through to below 0.5 mg. per daily dose. Urine contains X-substance. The crude extract is scarcely active, because it contains androsterone (with no hydroxyl group in the 17-position), and the activity of androsterone is not increased by the X-substance. The action of the X-substance is best demonstrated on the seminal vesicles of the rat; other animals are less well suited. X-substances occur in various natural products. Various known fatty acids, which increase the action of testosterone, occur in these same substances. Separate injections of testosterone and the X-substance fraction are less active than injection of a mixture of these substances. Probably resorption plays a part, but the activity of the X-substance cannot be explained by resorption alone (489, 490). Observations of the activating factor of acidic nature (X-substance) in extracts of testis and urine, led to preparation of the most varied esters of the male hormones, and to a detailed investigation into the question of the resorption of hormones. Certain esters prepared chemically show the same activity as that of a mixture of testosterone with X-substance (491). However, the

X-substance has not yet been identified chemically; it behaves like a highly unsaturated fatty acid or a mixture of such acids.

The most suitable method of extracting the comb-growth hormones in urine has again been investigated (492, 493). No destruction of the active substance occurs when the urine is hydrolyzed in the presence of benzene, because the liberated hormone passes directly into the organic solvent, and is thereby protected from the effect of the acid (492).

The color reaction with *m*-dinitrobenzene and potassium hydroxide in alcoholic solution, particularly in its modified and improved form, is very well suited to the determination of androsterone and other 17-ketosteroids, so that in the preliminary estimation of the male hormone content of urine, colorimetric determination may replace the biological assay (494). It has not yet been confirmed that an increased amount of estrogenic substances is excreted in the urine after administration of androsterone to male rats, or to men of different ages (495 to 497).

An excretion of male substances, in approximately the same quantities in men and women, has been observed in the urine of aged subjects, and of castrated women (498, 499), although the amounts eliminated are definitely smaller than in younger adults. Determination of the amount of male hormone eliminated has also a clinical significance (500). The amounts eliminated in cases of hypernephroma are enormously large (up to 1000 I.U. per liter of urine) (501).

An increase in the comb-growth activity of the blood was found after perfusion of bulls' testes (503). If dehydroandrosterone, androstenedione, and androstenediol were added to the blood before perfusion, a marked increase in the comb-growth activity was observed, which may be caused by conversion of the substances added into more active substances (503).

Testosterone propionate restores the normal structure of the accessory genital organs, and normal function in eunuch and castrated apes (490, 504 to 507, 519 to 521), and also in castrated rats (519). In young apes, testosterone propionate produced a premature descent of the testicles (508). These animals also show an increased secretion from the nasal mucous membrane, with congestion and swelling.

The reactions of the seminal vesicles of rats toward various male hormones is independent of the age and weight of the animal (509). This also holds if the height of epithelium is measured (510). The motility of the seminal vesicles of rats requires an adequate number

of smooth muscle fibers in the wall, and is inhibited by male hormones (517). The preputial glands of rats react to both male and female hormones (518, 511). Histological examination of the testes of individuals who have repeatedly committed offenses due to psychosexual aberrations, and who have been castrated by authority of the Danish Sterilization Act, has shown no morphological abnormalities; it may be suggested, therefore, that the testis is not the only site of sexual abnormality (512).

No alterations in acceleration of growth or maturation were observed from a histological examination of the testes of young monkeys after injection of testosterone propionate (513). Testosterone and various other male substances suppress the development of testicles in normal male rats (514); after hypophysectomy, testosterone propionate is able to maintain spermatogenesis (516). Teat growth in the male guinea pig is promoted by male hormones, particularly by trans-androstenediol (515). Treatment with testosterone propionate brings about the disappearance of the X-zone in normal adolescent males and females, adult females, castrated adult males, and adult ovariectomized females. X-zone formation has been prevented in young male and female mice (502).

An antimasculine action of the estrogenic hormones in male animals has been known for a long time. Further, it may be taken as certain that this antimasculine, or better, antitesticular action, proceeds through a suppression, by estrogenic substances, of hypophyseal function. An actual antagonism which brings about inhibition or suppression of action of the male hormone by the female hormone, has not yet been definitely proved. Also, the suppression in comb-growth activity of testosterone and androsterone by estrogenic hormones and by progesterone in capons (529, 530) probably proceeds through the hypophysis, although in a different way from the antitesticular suppression.

In castrated male mammals, estrone produces a definite increase in the weight of secondary sex organs—increase in the connective tissue, thickening of the smooth muscle, and proliferation of the epithelium—an action which is termed “paradoxical” (531). The “paradoxical” action on the tissues on which male sex hormones themselves have no exaggerating effect, can best be checked by the simultaneous administration of testosterone propionate (532, 533). These observations have given rise to the enunciation of a theory on prostate hypertrophy (534). Prostate hypertrophy is an expression of the male



climacteric; it is a disturbance in the function of the testis, during which the production of substances of the testosterone type declines, in the presence of undiminished amounts of estrogenic substances; this must have a "paradoxical" action (531). This theory provided the experimental basis for the therapeutic application of the male hormone in human prostate hypertrophy.

Prostate hypertrophy in dogs and mice is also beneficially affected by administration of gonadotropic hormones from pregnancy urine (535, 536). This agrees with the more recently established inhibition of "paradoxical" action in non-castrated mice by administration of gonadotropic substances from pregnancy urine (537, 538), which has been elaborated into a suitable therapy in the spontaneous prostate hypertrophy of dogs. The phenomena mentioned are best studied in these two kinds of animals. Other animals, in which similar "paradoxical" actions occur, are less suitable for the experimental study of prostate hypertrophy (539); however, rats may be used (540).

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## CHOLINE AS A DIETARY FACTOR\*

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The studies on the various lipotropic factors, *i.e.*, substances which prevent the deposition of fat in, or accelerate its disappearance from, the liver may be regarded as an outgrowth of certain aspects of insulin investigations. Large fatty livers were observed in depancreatized dogs maintained on insulin and certain mixed diets (1, 2). The addition of fresh beef pancreas to the diet prevented the development of the fatty livers. The first step in the identification of one of the factors responsible for this effect was taken by Hershey (3) and by Hershey & Soskin (4), who found that crude egg-yolk "lecithin" contained a factor which acted in the same manner as the fresh pancreas. These results were confirmed by Best & Hershey (5). It was then found that normal rats could be used as test subjects, and it was shown that purified lecithin was lipotropically active (6) and that choline was the active component of this phospholipid (7, 8).

### THE EFFECT OF CHOLINE ON THE DEPOSITION OF LIVER FAT

It was soon noticed that the mixture of grains used in the original diet for producing fatty livers in rats was rich in choline and possibly other lipotropic factors. In order to eliminate these factors, a ration consisting of a purified protein, carbohydrate (usually in the form of sucrose), mineral salts, vitamins A, D, and B<sub>1</sub>, and some easily available form of neutral fat was used when it was desired to produce the so-called "fat" fatty livers. In other experiments a diet composed essentially of sucrose with mineral salts and the vitamins possessed definite advantages. A third type of diet provided sufficient cholesterol to produce the "cholesterol" fatty liver.

The results which have been obtained might be presented in a great many different ways, but we have decided to classify them, insofar as

\* The preparation of a review of recent work on various aspects of choline research was originally intended, but more recently it was decided to restrict it to a consideration of the lipotropic action. While parts of this subject have been discussed previously in these *Reviews*, no comprehensive treatment has hitherto been attempted.

the effect of choline itself is concerned, on the basis of the methods which were used for the estimation of the fat content of the liver.

*Results based on the estimation of fatty acids plus unsaponifiable matter.*—Using this method of determining liver fat, a great many significant results have been obtained, but only those which have not been extended by the use of fractionation methods and those which are not to be discussed under other headings will be referred to here. It has been shown that the lipotropic action of choline is exhibited when variations in the protein, fat, and carbohydrate content of the diet are made (9, 10, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21). The deposition of liver fat resulting from the feeding of sucrose is also prevented by choline (12, 13).

Cedrangolo & Conte-Marotta (22) confirmed the lipotropic effect of choline on liver fat, but, as the diets contained large amounts of naturally occurring choline, the difference in amount of liver fat between the control and test group was small. Welch & Welch (23) have recently confirmed the findings of Best, Huntsman & Solandt (24) that choline chloride prevents the deposition of fat in the livers of mice receiving a diet rich in fat, and, using this species, they have elaborated a six-day method for the assay of lipotropic substances. Halliday (25) has noted fatty degeneration in livers of rats which received diets deficient in vitamin B<sub>6</sub>; the administration of choline did not entirely prevent the fatty change which occurred under the experimental conditions. Tanzi (26) observed that from four to ten days after birth the liver of the chick has a high fat content. Choline caused a marked decrease, which was accompanied by an increase in hepatic glycogen.

*The effect of choline on the glyceride fraction of "fat" and "cholesterol" fatty livers.*—In the first experiments in which liver fat was fractionated (27), it was shown that the extensive deposition of neutral fat which resulted from feeding diets high in fat or cholesterol was inhibited by choline. This was confirmed by Aylward, Channon & Wilkinson (28) who noted an effect of choline on the glyceride fraction after a single meal rich in fat. The preventive effect of choline has been confirmed in the fatty liver produced by the use of diets high in fat and containing varying amounts of protein and carbohydrate and also in the "cholesterol" fatty liver (10, 18, 19, 20, 29, 30, 31).

The lipotropic action of choline or other lipotropic factors is most easily shown when it is exerting its effect on livers with a high fat

content. It has been frequently observed that the maximum effect of added choline is elicited when it is added to diets containing a minimum of lipotropic factors.

Although small doses of choline do exert a marked action on liver fat, Channon, Loach & Tristram (20) found difficulty in differentiating between the effects of small successive decreases of the order of 5.5, 3.9, and 3.1 mg. daily. It was possible, however, to secure changes when amounts such as 2.3, 1.6, and 0.8 mg. were used. These amounts were nearer the minimal effective doses. The smallest dose was ineffective on the cholesterol type of fatty liver. In another experiment on the "fat" fatty liver it was shown that 0.7 mg. of choline daily did exert an effect. While small amounts of choline have a marked preventive effect on deposition of liver fat, larger quantities are necessary to maintain it at a normal level.

Choline also accelerates the removal of fat from fatty livers produced by feeding diets rich in fat (13). This action of choline has also been noted on the glyceride fraction of the fatty liver produced by cholesterol feeding (32, 33, 34).

*The lipotropic effect of proteins and amino acids.*—The first evidence that protein affected the deposition of fat in the liver was obtained by Best & Huntsman (13); see also Best & Channon (35). The addition of casein to the diet was found to reduce the amount of fat in the liver in a curative experiment. Since the lipotropic activity of casein was not due to the negligible amount of choline it contained, it was suggested that the action might be through formation of betaine from some of the amino acids contained in the protein. It was also appreciated that it might be due to a contaminant of the dietary casein. The lipotropic effect of 1 gm. of casein has usually been found to be equivalent to 5 to 8 mg. of choline (17, 29). Channon & Wilkinson (15) noted that the amount of glyceride in the "fat" and "cholesterol" fatty livers was governed by the amount of dietary casein. The Liverpool workers were the first to use diets low in protein as well as in choline for the production of fatty livers. Further studies of the lipotropic effect of casein on the deposition of glycerides in liver produced by feeding cholesterol or neutral fats have been reported (36). The lipotropic effects of casein and choline were compared. These workers suggested that the lipotropic effect of casein differed, at least superficially, from that of choline, in that with the former the absolute amount of phospholipid in liver rose with an increase in dietary casein. Best, Grant & Ridout (29) confirmed the lipotropic

action of casein on the glyceride fraction of "fat" fatty livers and found that a diet which contained 30 per cent casein exerted an optimum effect. The decrease in liver fat which is obtained by increasing the dietary casein is not the result of a corresponding decrease in the carbohydrate intake (17, 29). Fresh beef muscle extracted with alcohol was less active than casein and untreated egg albumin was essentially similar. Gelatin was found to have an insignificant lipotropic activity, and this may be due, in large part, to its low methionine content (37). Beeston, Channon, Loach & Wilkinson (17) reported that edestin was lipotropically active.

Channon and his collaborators (38) have studied the lipotropic activities of a number of plant and animal proteins on the "fat" and "cholesterol" fatty liver using casein as a standard. They found that commercial albumin increased the fat content of liver more than casein when fed at levels of 14 per cent or less, but when increased above this level a marked lipotropic action similar to that of casein was noted. Since the lipotropic action of proteins may be due to their constituent amino acids, these investigators emphasize the importance of the nature and amount of the protein in the basal diet in testing the activity of protein supplements. From these experiments they conclude that the lipotropic activities of proteins in order of decreasing intensity are as follows: whale-muscle proteins, caseinogen, albumin, beef-muscle protein and edestin, fibrin and gliadin, and lastly gelatin and zein. Best & Ridout (39) showed that under certain conditions dietary casein accelerated the disappearance of cholesterol esters as well as glyceride from the "cholesterol" fatty liver. ✓

While various investigators (40, 41) have previously shown that cystine produces fatty livers, Channon *et al.* (19, 42), using a diet containing a small amount of protein such as casein or egg albumin, have recently greatly extended these studies. Stimulated by the results of the latter workers, Tucker & Eckstein (43) fed methionine and found that it exerted an effect on liver fat which was just the opposite to that of cystine. These results have been confirmed (19, 44). Best & Ridout noted that under certain experimental conditions an increase in the dose of methionine failed to produce the expected further decrease in liver fat (44). Channon and his colleagues have shown that the effect of 20 and 40 mg. of methionine daily may be insignificant if the material does not act upon livers containing appreciable amounts of fat. Under other conditions, when the liver fat was 20 per cent or more, they concluded that the lipotropic activity of methionine was

about one-twelfth that of choline. If amino acids liberated during digestion behave in the same way as amino acids added to a diet, the lipotropic effect of dietary protein may be due to a balance between the factors accelerating and those preventing fat deposition. However, Channon and his colleagues do not believe there is sufficient evidence to show that the cystine and methionine contents of casein are entirely responsible for its lipotropic effect. They point out that it may be due, in part, to a contaminant. In more recent experiments, Tucker & Eckstein (37) have observed the lipotropic effect of dietary methionine when added as a supplement to gliadin which has a higher cystine content than casein. In contrast to the findings with casein, when gliadin was supplemented by cystine there was no further increase in liver fat. It is suggested that other amino acids may exert effects similar to cystine or methionine. Lysine was found to have little if any effect. Negative results of a preliminary study on various amino acids (lysine, glutamic acid, aspartic acid, serine, glycine, and phenylalanine) had been previously reported by Beeston & Channon (42). Tyrosine may exert a slight lipotropic action (45).

*The lipotropic effect of analogues of choline.*—The first substance other than choline shown to exert a lipotropic effect was betaine (8). Later experiments (44) showed that betaine was somewhat less active than choline. A number of other substances chemically similar to choline but without the hydroxyl group have been studied. Trimethylammonium chloride caused an increase in liver fat while trimethyl-ethyl-, tetramethyl-, and trimethylphenyl-ammonium chloride were extremely toxic (46). Welch (47) found that the arsenic analogue of choline was as effective as choline in preventing fatty changes in the liver and this finding has been confirmed (44). Channon and his coworkers tested the lipotropic action of certain analogues of choline and concluded that triethyl- $\beta$ -hydroxyethylammonium hydroxide probably had about two-thirds of the activity of choline while tripropyl- $\beta$ -hydroxyethylammonium hydroxide exerted an insignificant effect. Thus an increase in size of the alkyl radical of choline decreased its lipotropic activity. Trimethyl- $\gamma$ -hydroxypropylammonium hydroxide was found to have a greater lipotropic effect than choline (16, 18). Trimethyl- $\beta$ -hydroxypropylammonium hydroxide was shown to have no lipotropic activity (44).

Welch & Welch (48) have recently tested various choline derivatives by the mouse method of assay (23). The results of preliminary experiments by this method have indicated that N-betaine aldehyde



chloride, N-betaine hydrochloride and the phosphorus and arsenic analogues of choline chloride are more than one-half as active as choline chloride. The results with other derivatives are given qualitatively in the accompanying table (Table I).

TABLE I  
LIPOTROPIC ACTIVITIES OF VARIOUS COMPOUNDS

Compound	Formula	Lipo- tropic Activity	Refer- ence
Choline chloride .....	$(\text{CH}_3)_3\text{NClCH}_2\text{CH}_2\text{OH}$	+	(7)
Betaine aldehyde chloride ..	$(\text{CH}_3)_3\text{NClCH}_2\text{CHO}$	+	(48)
Betaine hydrochloride ....	$(\text{CH}_3)_3\text{NClCH}_2\text{COOH}$	+	(8)
Phosphocholine chloride ...	$(\text{CH}_3)_3\text{PClCH}_2\text{CH}_2\text{OH}$	+	(48)
Phosphobetaine hydro- chloride .....	$(\text{CH}_3)_3\text{PClCH}_2\text{COOH}$	—	(48)
Arsenocholine chloride ....	$(\text{CH}_3)_3\text{AsClCH}_2\text{CH}_2\text{OH}$	+	(47)
Arsenobetaine hydro- chloride .....	$(\text{CH}_3)_3\text{AsClCH}_2\text{COOH}$	—	(48)
$\beta$ -Methylcholine chloride ..	$(\text{CH}_3)_3\text{NClCH}_2\text{CH}(\text{CH}_3)\text{OH}$	—	(48)
$\alpha$ -Methyl- $\beta$ -phenyl- choline chloride .....	$(\text{CH}_3)_3\text{NClCH}(\text{CH}_3)\text{CH}(\text{C}_6\text{H}_5)\text{OH}$	?	(48)
Ethyl ether- $\beta$ -methyl- choline chloride .....	$(\text{CH}_3)_3\text{NClCH}_2\text{CH}(\text{CH}_3)\text{O.C}_2\text{H}_5$	—	(48)
Betaine aldehyde acetal chloride .....	$(\text{CH}_3)_3\text{NClCH}_2\text{CH}(\text{OC}_2\text{H}_5)_2$	+	(48)
$\alpha$ -Methylbetaine hydro- chloride .....	$(\text{CH}_3)_3\text{NClCH}(\text{CH}_3)\text{COOH}$	+	(48)
Calcium phosphoryl-cho- line chloride .....	$(\text{CH}_3)_3\text{NClCH}_2\text{CH}_2\text{O.PO}_3\text{Ca}$	+	(48)
Trimethylamine oxide hydrochloride .....	$(\text{CH}_3)_3\text{NCl.OH}$	—	(48)
Trimethylammonium chloride .....	$(\text{CH}_3)_3\text{NClH}$	—	(46)
Trimethylethylammonium chloride .....	$(\text{CH}_3)_3\text{NClCH}_2\text{CH}_3$	toxic	(46)
Tetramethylammonium chloride .....	$(\text{CH}_3)_4\text{NCl}$	toxic	(46)
Trimethylphenylammonium chloride .....	$(\text{CH}_3)_3\text{NClC}_6\text{H}_5$	toxic	(46)
Triethyl- $\beta$ -hydroxyethyl- ammonium hydroxide ....	$(\text{C}_2\text{H}_5)_3\text{NOHCH}_2\text{CH}_2\text{OH}$	+	(16)
Tripropyl- $\beta$ -hydroxyethyl- ammonium hydroxide ....	$(\text{C}_3\text{H}_7)_3\text{NOHCH}_2\text{CH}_2\text{OH}$	+	(18)

TABLE I (Continued)

Compound	Formula	Lipo- tropic Activity	Refer- ence
Trimethyl- $\gamma$ -hydroxypropyl- ammonium hydroxide ....	$(\text{CH}_3)_3\text{NOHCH}_2\text{CH}_2\text{CH}_2\text{OH}$	+	(18)
Trimethyl- $\beta$ -hydroxypropyl- ammonium hydroxide ....	$(\text{CH}_3)_3\text{NOHCH}_2\text{CHOHCH}_3$	—	(44)
Methionine .....	$\text{CH}_3\text{SCH}_2\text{CH}_2\text{CHNH}_2\text{COOH}$	+	(43)
Ergothioneine .....	$\left[ \begin{array}{c} \text{HC} \text{---} \text{N} \\ \parallel \quad \diagup \quad \diagdown \\ \text{C} \text{---} \text{NH} \quad \text{C} \text{---} \text{SH} \\   \\ \text{CH}_2 \\   \\ \text{HC} \text{---} \text{N}(\text{CH}_3)_3 \\   \\ \text{OC} \text{---} \text{O} \end{array} \right]$	—	Authors' unpub- lished observa- tions

*The effect of choline on deposition of liver fat produced by non-dietary means.*—Choline is very effective in preventing glyceride deposition or accelerating glyceride removal in those cases in which deposition is produced by dietary means. One of the principal difficulties in these studies is that many species of animals cannot be made to ingest adequate amounts of the diet low in choline.

The finding that the glyceride fraction of the "cholesterol" fatty liver is less affected by a given dose of choline than is the same fraction in the "fat" fatty liver may be due to the fact that the cholesterol causes a certain amount of liver damage: in other words, the cholesterol acts as a mild toxic agent.

Choline does not inhibit the deposition of fatty acids in the acute fatty livers produced in rats by phosphorus poisoning (49, 50) or the fatty liver produced by injections of anterior pituitary extract (51, 52). The latter finding was confirmed in rabbits by Mukerji & Guha (53). Choline does not inhibit the increase in liver fat in rabbits following a single large dose of pitressin (54).

Cedrangolo & Conte-Marotta (55) claim that the administration of choline inhibits the deposition of fat in livers of rats after fasting and phlorhizin poisoning. Laszt & Verzár (56) have made a similar claim for the action of choline in rats poisoned by phosphorus. In both these investigations conclusions are based on extremely small changes in liver fat. In fasting and in phlorhizin-poisoned dogs, how-



ever, choline apparently exerts a definite preventive action on deposition of liver fat (57).

It must be remembered in this connection that when the deposition of fat in liver is produced by toxic agents such as phosphorus, and the administration of the agent is discontinued, the rate of disappearance of fat from the liver in the recovery period is accelerated by choline (49, 56).

*Choline and the fatty liver of starvation.*—It has been reported that large doses of choline given for long periods before and during fasting prevent the accumulation of fat which may take place in the liver of the rat (58). With amounts of choline which come within the physiological range, however, no effect on the fatty liver of starvation in guinea pigs, mice or rabbits has been observed. In fasting female rats previously fed a normal diet and with large deposits of depot fat, choline may exert a slight effect on the liver fat which accumulates under these conditions (14). On the other hand, it has been claimed (21) that rats which have been on a high-fat diet with choline for fourteen days, deposit a labile fat in their depots. In a subsequent fasting period of three to five days there is a considerable increase in liver fat. This increase is prevented if the administration of choline is continued. It has been shown that during fasting choline accelerates the disappearance of fat from the livers of animals in which fatty livers have been produced previously (14, 21, 22). Choline has no effect upon the deposition of fat which occurs during fasting in rats upon which a partial hepatectomy had been performed (59, 60).

#### CHOLINE AND THE METABOLISM OF CHOLESTEROL

It is well known that the addition of cholesterol to the diet of rats results in accumulation of cholesterol esters and neutral fat in the liver. The first evidence that lipotropic factors inhibited deposition of fat due to cholesterol feeding was obtained by Best & Ridout (61). Both choline and betaine were effective. The results suggested that the unsaponifiable fraction as well as the glyceride had been reduced. In later experiments the liver fat was fractionated (27) and definite evidence was obtained that choline inhibited the deposition of cholesterol esters as well as glyceride. This finding was confirmed and it was noted that the effect on the glyceride was more marked than on the cholesterol esters (36). In studies on the curative effect of choline on the "cholesterol" fatty liver Channon & Wilkinson (32)

showed that choline had no obvious effect in twelve days in causing a decrease in the lipid constituents except perhaps in the case of the glyceride fraction. It was later shown (33) in more prolonged experiments on rats which had a high cholesterol ester content in the liver that the administration of choline hastened the disappearance of cholesterol esters. When a small dose of cholesterol was continued after fatty livers had been produced by cholesterol feeding, the addition of choline caused a definite fall in cholesterol esters as well as in the glyceride in eighteen days. It was also noted that when larger doses of cholesterol were continued during the test period a delayed effect of choline on the esters was observed in prolonged periods (34). Stoesser, McQuarrie & Anderson (31) found that choline partially inhibited the deposition of cholesterol in the livers of cholesterol-fed rats and prevented the accumulation of glyceride. The evidence from all these papers suggests, but does not prove, that the effect of choline is exerted first upon the glyceride fraction and subsequently upon the cholesterol esters. It is not the case, however, that the glyceride content must be reduced to a very low level before an effect upon the cholesterol esters can be observed. Larger doses of choline are required to affect cholesterol esters than glycerides.

Baumann & Rusch (62) reported that choline failed to inhibit the deposition of cholesterol and glyceride in the livers of cholesterol-fed rabbits but their figures suggest that the extremely high level of cholesterol intake may have been responsible for these findings. Hims-worth (63) has noted that large doses of choline may inhibit the deposition and accelerate the removal of unsaponifiable matter from the livers of cholesterol-fed rabbits.

*Protein and the "cholesterol" fatty liver.*—The lipotropic action of protein on the deposition of cholesterol esters was first studied by Channon & Wilkinson (15). These investigators [later in collaboration with Beeston (36)] reported that protein had no lipotropic effect on the cholesterol-ester fraction of liver fat. They realized, however, that their results might be attributed to an insufficient amount of protein since the choline equivalent of the protein fed might not have exerted any definite effect on the cholesterol esters. However, when a moderate deposition of cholesterol esters was produced in livers of rats, definite evidence that the non-choline lipotropic factor in dietary casein accelerates the disappearance of cholesterol esters from liver was reported by Best & Ridout (39). The magnitude of the effect produced by casein on cholesterol esters was therefore essentially that

which would have been expected from its choline equivalent. Similar results have been noted previously for the glyceride fraction.

Channon, Manifold & Platt (19) have shown that the administration of methionine inhibits the deposition of cholesterol esters in liver.

Channon, Platt & Smith (18) tested the action of the following analogues of choline on the deposition of cholesterol esters: triethyl- $\beta$ -hydroxyethylammonium hydroxide, tripropyl- $\beta$ -hydroxyethylammonium hydroxide and trimethyl- $\gamma$ -hydroxypropylammonium hydroxide. In general it may be said that the relative effects of these substances on cholesterol esters are the same as upon the glyceride fraction of liver fat. No evidence has been obtained that any of the choline analogues exerts correspondingly more effect on cholesterol esters than on the glycerides.

*The effect of choline on blood cholesterol and experimental atherosclerosis.*—It has been demonstrated by Steiner (64), Baumann & Rusch (62) and Himsworth (63) that choline has no effect on the marked hypercholesterolemia of cholesterol-fed rabbits. Baumann & Rusch, and Himsworth have reported that choline has no effect on the cholesterol deposition in the aorta of cholesterol-fed rabbits. Steiner (64), from a macroscopic examination, concluded that choline delays but does not prevent atherosclerosis in rabbits. More recently (65) he has obtained results which suggest that choline hastens reabsorption of the atheromatous plaques.

#### CHOLINE AND KETOSIS

MacKay & Barnes reported that in fasting rats choline has a slight effect on the ketonuria which accompanies the fatty liver produced by anterior pituitary extract. The pancreatic extract (lipocaic) was without effect (52). Deuel, Murray, Hallman & Tyler (21) have, however, demonstrated a more definite effect of choline upon ketosis. The level of ketonuria was found to be lower on the first two days and greater on the next three days of fasting in rats which had previously received a diet rich in fat plus choline than it was in the control animals. The administration of choline to fasting rats which had previously received a diet rich in fat plus choline lowered the ketone body production. The administration of choline during the fasting of rats which had previously received a high-fat diet without choline did not affect the ketonuria until the fourth or fifth day when it was lowered significantly. These very interesting findings demand further investigation.

## THE QUESTION OF A LIPOTROPIC FACTOR IN PANCREAS OTHER THAN CHOLINE AND PROTEIN

While the results obtained by the Toronto group (66) definitely proved that choline is one of the factors in pancreas which prevents the deposition of fat in the livers of depancreatized dogs, it was never claimed that this was the only one present. Indeed, after the demonstration by Best & Huntsman (8) that betaine was lipotropically active, and the findings by the same authors that protein exerted a lipotropic effect, it appeared extremely probable that some of the lipotropic action of pancreas might be due to these or closely related factors (58). It remained for Dragstedt, Van Prohaska & Harms (67) to claim that a new pancreatic hormone which they named "lipocaic" had been demonstrated. The fat content of small pieces of the liver of depancreatized dogs was studied microscopically at various intervals before and after the administration of the pancreatic extract and the results suggested the presence of a new factor which inhibited deposition of fat in, and disappearance of fat from, the liver. While they observed a lipotropic action with large doses of choline they concluded that there was not sufficient choline in the extract to account for the effect observed. These results have been criticized by Chaikoff & Kaplan (68) who threw some doubt on the method used by Dragstedt and his collaborators in sampling the livers, and by Ralli, Rubin & Present (69) who noted fatty livers in dogs whose pancreatic ducts had been tied. This latter finding is not in agreement with Dragstedt's hypothesis of an internal secretion of the pancreas which controls deposition of liver fat. Kaplan & Chaikoff<sup>1</sup> (70), Ralli and her collaborators (71) and Van Prohaska, Dragstedt & Harms (72) have confirmed the findings of Best, Ferguson & Hershey (66) that large doses of choline prevent deposition of fat in the livers of depancreatized dogs. More recently Chaikoff & Kaplan (73) have noted a prophylactic effect with much smaller doses (100 mg. daily). They have shown that the pancreatic factor which causes a rise in blood cho-

<sup>1</sup> These workers have reported that depancreatized dogs treated with insulin may survive for long periods without dietary supplements of lecithin or choline. These results have been repeatedly cited by reviewers as evidence that choline is not essential for the survival in good condition of depancreatized dogs. The correct interpretation of the findings is, as Chaikoff & Kaplan point out [*Proc. Soc. Exptl. Biol. Med.*, 33, 211 (1935); *J. Nutrition*, 14, 459 (1937)], that the diets used (560 gm. of lean beef daily, etc.) contained sufficient choline and other lipotropic factors to make supplements of these materials unnecessary.

lesterol in depancreatized dogs is not choline (70). Aylward & Holt (74) fed raw pancreas to rats and found no evidence that the effect of the pancreas was due to anything in addition to its choline content. MacKay (75) and MacKay & Barnes (76) have prepared a pancreatic extract by Dragstedt's procedure and have tested it for its effect on deposition of fat in the livers of rats. In the more recent paper, they report the presence of a much greater amount of lipotropic substance than is found in casein or muscle protein, but they find no evidence that a factor in addition to protein and choline exists. Best & Ridout (12) using normal rats tested raw pancreas and a sample of lipocaic made by Eli Lilly and Co. and have concluded that their lipotropic activity is due to choline and protein. On the other hand, Shapiro & Wertheimer (77) and, more recently, Channon, Loach & Tristram (20) using normal rats claim that they have demonstrated the presence of a non-protein lipotropic factor other than choline in extracts of pancreas. The data of the former group have not been published in detail. Channon and his collaborators have prepared pancreatic extracts by a procedure which apparently differs considerably from that first used by Dragstedt since many of their preparations contain large amounts of choline. Goodpasture, Vermeulen, Donovan & Dragstedt (78) have confirmed the previous findings from their laboratory with regard to the effect of lipocaic on liver fat in depancreatized dogs and have suggested that the results of the bromsulphalein test of liver function may provide an additional criterion of lipocaic deficiency. We have recently tested the effect on the deposition of liver fat in rats of very small doses of another lipocaic preparation (containing 0.15 per cent choline) made by Eli Lilly and Co. and fail to find more than a small amount of non-choline lipotropic activity. Whether or not this is due entirely to the pancreas protein can be determined only after the protein is isolated or perhaps after the amino acid content is determined. The choline equivalent of the total lipotropic activity of 1 gm. of this lipocaic is not more than 10 mg. Dragstedt and his collaborators obtained an effect in the depancreatized dogs by feeding from 1.0 to 1.5 gm. of lipocaic daily. As we have suggested previously, these figures indicate the presence in lipocaic of some factor affecting liver fat in depancreatized dogs but not in normal rats.

Huber, Broun & Casey (79) state that lipocaic prevents the rise in blood cholesterol and the development of atherosclerosis in the aorta which regularly occurs in rabbits fed a diet rich in cholesterol. The

effect of lipocaic on the alcoholic fatty liver in dogs is still indefinite [Snell; see Rosenberg (82)].

Grayzel & Radwin (80) reported a marked reduction in liver size in three young diabetics with hepatomegaly when a pancreatic extract (lipocaic) was fed. Positive results in clinical cases of hepatomegaly have been claimed by Snell & Comfort (81) and by Rosenberg (82). White, Marble, Bogan & Smith (83) have not observed any definite effect on the hepatomegaly of juvenile diabetics as a result of feeding either raw pancreas or betaine. Rabinowitch (84) has suggested that betaine administration may improve liver function in certain diabetic patients.

We feel that the fatty liver of diabetic patients is much more likely to be due to inadequate insulin therapy than to a deficient supply of lipotropic factors, *i.e.*, to increased transport of fat to the liver from the depots rather than decreased ability of the liver to pass fat on to the depots. The fact that most human diets contain large amounts of the lipotropic factors makes clinical investigations along these lines very difficult.

#### THE MODE OF ACTION OF CHOLINE

It is now well established that choline in the diet inhibits the accumulation of neutral fat and cholesterol esters in, and accelerates their disappearance from, the liver. The use of diets poor in choline but more nearly adequate in other respects than those used in the early experiments has shown that choline favors the normal distribution of dietary fat, *i.e.*, prevents accumulation of fat in the liver but encourages deposition in the depots (11). Deuel, Murray, Hallman & Tyler (21) reached a similar conclusion from their studies of the effect of choline on ketosis. This distribution of fat may be an important factor in the gain in weight produced by choline under certain conditions (10). McHenry (85, 86) has shown that choline and thiamin exert complementary effects upon the increase in weight of young rats. This effect is less marked in older rats. Furthermore, the use of diets low in choline has enabled him to prove that the administration of thiamin to rats accelerates the deposition of fat in the livers. When a large dose of choline and an amount of thiamin in excess of that necessary to prevent polyneuritis are present in a diet, the effect of the former predominates insofar as liver fat is concerned. It has been pointed out by Williams & Spies (87) that choline and thiamin possess some chemical similarity in that they are both quaternary

bases and both are derivatives of ethyl alcohol. An unidentified component of rice polishings, possibly vitamin B<sub>6</sub>, may also play a rôle in the transfer of fat from liver to depots (88).

There is evidence which indicates that lack of choline and related factors results in partial loss of liver function. When diets deficient in choline and related factors are supplied to depancreatized dogs, fat not only accumulates in the liver but there is a failure of the gluconeogenic mechanism (3, 4, 5, 66). When normal rats receive a diet deficient in lipotropic factors for long periods the liver tissue loses some of its ability to store glycogen and to excrete dye (11). If the accumulation of excess fat in the liver also be considered a sign of failure of hepatic function, the action of choline and related substances as accessory food factors appears to be, in large measure, the preservation of this function. The question arises as to whether the accumulation of fat produces the failure of function, or is the deposition of fat a result of failure of function?

The finding that the amount of choline contained in highly purified lecithin (7, 8) exerted approximately the same lipotropic effect as the lecithin itself, suggested either that the effect of the phospholipid was due to the choline liberated from it or that the choline acted by forming lecithin. It appears that lecithin is synthesized in the body when the components are available in the diet (89, 90, 91, 92) but a deficiency of choline and related factors in the diet makes itself apparent, insofar as liver fat is concerned, within a few days or even much sooner (28) in spite of the presence in the body of very large amounts of choline-containing phospholipids. The choline of lecithin, of sphingomyelin, or of other choline-containing compounds is therefore not available (93, 94, 95, 96, 97). There is no evidence that there is a decrease in the total phospholipid content of the liver when animals are maintained on a ration rich in fat and relatively poor in choline or that the provision of choline causes a significant increase (27, 36). The phospholipid is, of course, greatly diluted by the large amount of neutral fat which accumulates when the diet is deficient in lipotropic factors and this might conceivably produce an upset in physicochemical relationships. On the other hand, it may be argued that the failure to detect an increase in the total phospholipid content of liver on feeding choline does not preclude the possibility that there is an increased rate of formation which might make possible the more rapid and extensive transport or utilization of liver fat. Until very recently no direct evidence that dietary choline becomes a part of the



phospholipid molecule was available, but the use of an identifiable lipotropic factor, other than choline, offered an opportunity for testing this hypothesis. This ingenious experiment was first made by Welch (47) who fed arsenocholine chloride to rats and was able to demonstrate in his experimental animals but not in controls strong arsenic lines in the purified lecithin of brain and liver and in the sphingosine-phosphorylcholine of the kidneys. Channon, Platt, Loach & Smith (30), using triethyl- $\beta$ -hydroxyethylammonium hydroxide, which can be separated from choline by the lower solubility of its chloroaurate, found none of this material in the liver lecithin although a very definite lipotropic effect had been exerted. Very recently Welch & Welch (48) have reported further experiments, using mice instead of rats, the results of which have confirmed and extended their previous findings. Arsenic was found in the lecithin of the animals fed arsenocholine but not in those which received arsenic pentoxide. The hypothesis that choline chloride is phosphorylated and utilized in the synthesis of lecithin is supported by the fact that the phosphoric acid ester of choline chloride is unaffected by liver phosphatase and therefore protected from hydrolysis in the liver. The possibility that phospholipid is formed and transported to other tissues or utilized, in part, in the liver itself is now therefore an attractive one (27, 36, 47, 98, 99).

Let us for the moment explore other possibilities. Various hypotheses have been put forward. Many of these are not incompatible with the one outlined above. Best, Channon & Ridout (27) suggested that choline, either directly or indirectly, may accelerate the oxidation of fat in the liver itself. The prevention of fat accumulation may be linked with gluconeogenesis, but the evidence on this point is not yet definite (66, 100). Raab & Strauber (101) found that normal dogs on a standard diet and receiving choline by mouth showed an increase in blood sugar and ketone bodies but no change in blood fats. The most pronounced increase in ketone bodies was observed after choline had been discontinued. In human subjects the findings were similar but less pronounced. While these authors suggest that the intrahepatic new formation of sugar from fat may be the cause of the observed changes, neither they nor the present reviewers feel that this is the only explanation possible. Cedrangolo & Conte-Marotta (55) and Cedrangolo (57) have reported that the administration of choline during fasting to rats or dogs with fatty livers produced by phlorhizin causes an increase in liver glycogen which suggests a transformation of fat into carbohydrate. Tanzi (26) noted that the lipo-



tropic effect of choline was accompanied by an increase in hepatic glycogen. Ardy & Gallo (102) reported that the administration of choline to depancreatized dogs caused large increases in the reducing power of the liver during aseptic autolysis. Livers from depancreatized dogs which were not treated with choline failed to show an increase in reducing power under the same conditions. These authors concluded that the extra reducing substances did not come from fat since the fat content was not definitely affected. Cedrangolo & Conte-Marotta (22) have suggested that choline acts through the vegetative nervous system but their conclusions, based on the results of atropine and epinephrine administration to rats, are not convincing. Verzár (103) has also suggested this possibility. It has been reported (104) that the lipase of liver tissue is increased as a result of choline administration.

Several groups of investigators have studied the effects of the addition of choline to liver slices, pulp, or extracts. It was reported by Bernheim & Bernheim (105) that the choline fraction of acetylcholine was oxidized by liver pulp. In later work (106) they concluded that the effect of the liver enzyme on choline was influenced by the hydrogen ion concentration. Experiments conducted in an acid medium (pH 6.7) indicated that betaine aldehyde was formed and by adjusting the pH to 7.8 the oxidation proceeded with the production of betaine. However, these substances were not isolated. Trowell (107) provided evidence of the oxidation of choline by liver slices. He noted an inhibition of acetoacetic acid production (the end-stage of fatty acid oxidation under these conditions) when choline was added and concluded that choline decreased the rate of fat oxidation in the liver. Very large amounts of choline were used and the physiological significance of these findings is debatable. Trowell did not measure residual choline but this has been done by Mann & Quastel (108) who isolated the oxidation product in the form of the reineckate and aurichloride. They have shown that the increase in oxygen uptake when choline is added to rat liver slices or extract is due to the oxidation of choline to betaine aldehyde. More recently Mann, Woodward & Quastel (109) found that arsenocholine is also oxidized by liver slices to arsenobetaine aldehyde. It is suggested that the aldehyde may be oxidized further to betaine. Preliminary observations by Welch & Welch (48) indicate that N-betaine aldehyde is lipotropically active but they feel that the oxidation of choline to betaine aldehyde and betaine is probably not concerned with its lipotropic action. N-betaine

hydrochloride is active (8) but Welch & Welch have noted that the phosphorus and arsenic betaines are inactive while the corresponding cholines are active. They suggest therefore that N-betaine becomes active through conversion to choline.

It would appear that a comparison of the biochemical activities of liver slices from animals which have been maintained on diets poor in choline and lipotropic factors with those of animals on the same diet plus choline provides a better opportunity of investigating the physiological action of the base than does the addition of quite unphysiological amounts of the substance to liver tissue. Welch, Irving & Best (110) found that slices of fatty liver from animals on the diet poor in choline utilized less oxygen than those from the choline-fed rats. This is easily understood if we assume that the stores of fat are not "active tissue." Basing their calculations on "non-fatty dry material," the above authors felt that their figures suggested a loss of oxygen consumption of the liver tissue when choline was withheld. More recently, Woodward (111) has made an extensive investigation of this point. He found little or no difference in the  $Q_{O_2}$  of liver slices calculated on a "non-fatty dry material" basis between the "choline and non-choline" groups of rats until the experiment was extended for more than the usual three weeks. When the period of observation was prolonged there appeared to be no doubt that a loss of oxygen consumption, calculated on a "non-fatty dry weight" basis, could be clearly demonstrated in the liver slices from some but not from all animals whose diets had been very low in lipotropic factors. This brings us back again to the problem of whether the accumulation of fat causes the loss of function or whether the loss of function is the primary disturbance.

The simplest explanation of the action of choline is that its administration produces a very slight increase in the phospholipid content of the liver and that this promotes the transport of fatty acids, as phospholipids, from the liver to other tissues or utilization in the liver itself. It may not be necessary to postulate an increase in phospholipid in order to account for the accelerated transport or utilization of liver fat. There is perhaps no great difficulty in supposing that an increase in the rate of transfer of phospholipid from liver to depots would play a part in the disappearance of cholesterol esters as well as neutral fat from the liver. At one time we felt that this was unlikely (27).

The question arises as to whether choline is supplied in sufficient

amounts to form enough phospholipid to transport the fatty acids which are removed from the livers of the choline-fed animals, *i.e.*, the amount which accumulates in the livers of the animals which have not received choline. If one calculates the number of molecules of fatty acids which have accumulated in the livers of the animals on the diet low in lipotropic factors and postulates that one molecule of choline is necessary for the transport of two molecules of fatty acid, ample choline has been supplied to explain the absence of fat in the livers of the test animals. It should be appreciated that under certain circumstances a mechanism exists for the removal of neutral fat or cholesterol esters from the liver when lipotropic factors are not supplied in the diet (14, 21, 22, 33), *e.g.*, when rats with fatty livers are fasted the fat content of the liver decreases.

As a working hypothesis to explain the production of fatty livers on diets low in choline or related factors, it might be postulated (a) that, in the absence of sufficient amounts of choline to form phospholipid at the rate required to maintain the liver free of excess fat, neutral fat accumulates, and (b) that the presence of excessive amounts of fat for long periods of time is responsible for the decrease in liver function. On this hypothesis the only direct result of the lack of lipotropic factors would be the accumulation of fat. The failure of the mechanism for storing glycogen, excreting dye, making dextrose, and consuming oxygen would be attributable to the excess of fat, *i.e.*, only indirectly to lack of choline. An alternative hypothesis for which at the moment there is less evidence is that, in the absence of dietary choline and other lipotropic factors, all functions of the liver are disturbed but loss of ability to remove fat is the most readily apparent. Further investigations are necessary before a final answer to the question of whether either of these hypotheses is adequate or if some new one is required to explain completely the action of the lipotropic factors.

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## THE WATER-SOLUBLE VITAMINS

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### VITAMIN B<sub>1</sub> (THIAMIN)

Progress in the study of vitamin B<sub>1</sub> has continued rapidly, with special emphasis being placed upon (a) its function as cocarboxylase, and the related basic problems in tissue respiration and fermentation, (b) the growth-stimulating effect of the vitamin and its constituent units in plants, and (c) the relation of vitamin intake to health problems, with special reference to "alcoholic polyneuritis" in adults, and general nutrition in children.

*Methods of assay and analysis.*—Advances in methods of analysis and assay have consisted of varying degrees of modification and refinement in methods previously described. Schultz, Atkin & Frey (1) found that the yeast fermentation test for thiamin could be rendered more specific by the addition of 1 mg. of nicotinic acid to both control and test flasks. The test was further modified for urine analysis by the oxidation of thiamin to thiochrome (with ferricyanide) and noting the *decreased* activity after oxidation. This modification was not observed to be necessary in the assay of foodstuffs, but was made necessary in urine tests by the presence of other stimulants that were not destroyed by oxidation.

Schopfer's mold-growth test was used satisfactorily in a number of laboratories (2). Robbins & Kavanagh (3) pointed out, however, that closely related compounds, e.g., 2-ethyl-5-bromomethyl-6-aminopyrimidine may give a positive response analogous to that of thiamin with *Phycomyces blakesleeana*. West & Wilson (4) also obtained satisfactory assays with *Staphylococcus aureus*. It is obvious that a certain hazard is involved in the assay methods with lower plants, due to their greater capacity for synthesizing thiamin from simpler pyrimidine and thiazole units.

The catatorulin test, based upon the oxygen uptake of vitamin-deficient pigeon brain in the presence of dilute pyruvate solution was modified by Peters (5) who believes that the test will continue to be of value in quantitative studies of thiamin in quantities as low as 0.2 µg.

Arnold & Elvehjem (6) made a careful study of the vitamin B<sub>1</sub> requirement of the chick, in which a diet of autoclaved corn 57, auto-



claved middlings 25, autoclaved crude casein 12, vacuum-dried liver 2, iodized salt 1, calcium carbonate 1, tricalcium phosphate 1 and cod-liver oil 1 part was considered to provide a more adequate diet than is generally used for B<sub>1</sub> assays. The protective dosage of adsorbate was 20 to 25 International units per day. The protective level of the crystalline vitamin was 80 to 100 mg. per 100 gm. of ration.

A second paper by the same authors (7) described a ration that is more suitable than those generally used for measuring the thiamin requirement of rats, in that better provision is made for the other B factors: sucrose 62, purified casein 18, autoclaved peanuts 10, autoclaved brewer's yeast 10, salt mixture 4, liver extract (with factor W) 2 per cent, and 2 drops of halibut-liver oil twice weekly. Normal growth resulted when the diet was supplemented with 80 to 100 µg. of thiamin hydrochloride per 100 gm. of ration.

A paper by Light & Cracas (8) was of special interest in that they found evidence of marked differences in B<sub>1</sub> requirement that were due solely to differences in strain of rats. One of the three strains tested showed a growth response that was only equivalent to half the response of the other two strains.

A short-period rat curative assay technique was described by Kline, Tolle & Nelson (9), based on the earlier procedure of Smith, in which the following diet was found to be satisfactory: sucrose 61.25, purified casein 18, salt mixture 4, cod-liver oil 2, autoclaved yeast 4, autoclaved peanuts 10, purified liver extract 0.75 per cent. The diet was sufficiently adequate to permit repeated tests with the same animals. The use of sulfite treatment of the basal ration for the destruction of any thiamin present (Williams' reaction) was also suggested.

The rat-growth method of assay was found by Pedersen (10) to be approximately twice as accurate as the bradycardia method, but the latter was preferred for general use because of its greater simplicity. The thiochrome method was also found to be less accurate than the growth test. Supplee *et al.* (11) discussed briefly their experience with attempts to evaluate the accuracy of tests based upon growth rate compared to the incidence and cure of polyneuritis. A good review of the methods of assay and the distribution of vitamin B<sub>1</sub> in food products was prepared by Munsell (12).

The adoption of a new International unit, equal to 3 µg. of pure thiamin hydrochloride, should distinctly simplify the problem of having a standard reference material.

The most widely used chemical method for determining thiamin has been dependent upon the following sequence of steps: extraction in acid solution, adsorption upon fuller's earth, frankonite, or similar material, elution in alkaline solution, oxidation to thiochrome (generally by means of ferricyanide) and measurement of thiochrome concentration by its blue fluorescence. Westenbrink & Jansen used this method extensively with apparent success (13) for analyzing a variety of rat tissues, 10 cc. blood samples, and urine. After oxidation the free thiochrome could be separated from an alkaline solution into isobutanol, leaving the pyrophosphate derived from cocarboxylase in the aqueous layer. In rat tissue nearly all of the thiamin was present as cocarboxylase (14). Roth also used the method with apparent success for the determination of free thiamin, cocarboxylase, and the monophosphate ester—the latter being differentiated from the coenzyme on the basis of splitting off carbon dioxide from pyruvic acid. In addition to general use of the method for tissue analysis, Otto & Rühmekorb (15) studied the range of concentration, light intensity, and exposure time that could be used satisfactorily with the step-photometer. Euler & Willstaedt obtained very low results with the fluorescence method, but found fairly good agreement between the colorimetric diazo method and animal assays (16). Vitamin-deficient rat muscle contained less than half as much vitamin as normal muscle. Melnick & Field (17) studied the method of Prebluda & McCollum, in which the adsorbed and eluted thiamin is coupled with diazotized *p*-aminoacetophenone. Cocarboxylase was converted to free thiamin enzymically before developing the color.

An adaptation of the formaldehyde azo reaction for determining thiamin in foodstuffs and tissues was studied by Kinnersley & Peters (18). They also used an enzymic hydrolysis to differentiate the free vitamin from cocarboxylase. Raybin proposed a color reaction with 2,6-dibromoquinone-chlorimide (19), and Slotta & Neisser proposed a direct titration with 0.01 to 0.05 *N* hypoiodite, followed by back titration with thiosulfate (20).

The reducing action of thiamin, in comparison with nicotinic acid-amideethiodide and three quaternary thiazoles by hyposulfite and by hydrogen with palladium black, was studied by Lipmann & Perlmann (21).

Two reviews of the chemistry of vitamin B<sub>1</sub> were prepared by Williams during the year (22), in addition to the publication of a book, jointly with Spies (23).

*Occurrence in foods.*—An extensive study of the vitamin B<sub>1</sub> content of fish products was carried out by Lunde, Kringstad & Olsen (24). Both chemical and biological assays were made, with the following results, expressed approximately in International units per 100 gm.: cod roe 411, cod liver 100, other fish livers 50 to 100, fish flesh 20. Canning destroyed less than 50 per cent of the B<sub>1</sub> content of cod roe.

Donath & Spruyt (25) published a summary of their work on the thiamin content (by biological assay) of a great variety of East Indian vegetables, which will be of general interest as a reference.

The increased vitamin B<sub>1</sub> content of yeast as a result of (a) growing without aëration, (b) supplying grain wort, or (c) adding vitamin B<sub>1</sub>, liver extract or nucleic acid to the medium, was shown by Pavcek, Peterson & Elvehjem (26). Arnold & Elvehjem observed very little loss (up to 20 per cent) in the B<sub>1</sub> content of meat products as a result of canning and storing for two years (27). Values obtained for the thiamin content of vacuum-dried products, expressed in International units per gram, were: beef kidney 5, beef spleen 2, beef lung 2, hog brains 1.6.

In a study of commercial meals, Sherwood & Halverson obtained values ranging from 1.1 to 5.4 International units per gram (28).

*Coccarboxylase synthesis.*—Using the thiochrome method of analysis referred to previously, Westenbrink & Goudsmit (29) made a careful study of the thiamin and coccarboxylase content of rat tissues after a three to four weeks period of avitaminosis, and the rise in both substances after an injection of 2 mg. of thiamin. The depleted animals showed only an extremely small amount of free vitamin, and slight amounts of coccarboxylase in any of their tissues. Within a few minutes after injection there was a marked rise in the coccarboxylase content of the liver, followed by a slow rise, after about a half-hour, in the kidneys. No distinct, rapid rise was observed in the other tissues. The tissues studied were liver, muscle, kidney and brain, in rats, pigeons and rabbits.

Enzymic synthesis of coccarboxylase *in vitro* was demonstrated and discussed, particularly with reference to kinetics and inhibitors, by Lipschitz, Potter & Elvehjem (30). Synthesis occurred readily in the presence of washed dried yeast with hexosediphosphate and boiled tissue extract. Iodoacetate and fluoride ion served as inhibitors, the fluoride ion inhibition being offset by added pyruvate, especially when working with an atiozymase preparation instead of washed

yeast. The authors concluded that cocarboxylase synthesis was linked with the dismutation between triosephosphate and pyruvic acid.

Tauber prepared pure cocarboxylase by direct synthesis, heating thiamin with phosphoric acid and sodium pyrophosphate at 155°, followed by crystallization (31). Synthesis was also accomplished by enzymes from yeast and duodenal mucosa (32). Activating effects upon cocarboxylase were observed with sodium cyanide, magnesium chloride, magnesium sulfate, sodium chloride, potassium chloride and sodium sulfate. A method for preparing crude cocarboxylase from yeast was described by Kinnersley & Peters (33). Ochoa & Peters (34) reported that thiaminmonophosphate exerted an increased activity on cocarboxylase in alkaline washed yeast, acting upon pyruvate. Hexosediphosphate also increased the activity of cocarboxylase in the presence of cozymase, without the presence of thiaminmonophosphate. The authors noted the marked activating effect of manganeous ion on cocarboxylase. Estimates of thiaminmonophosphate in rat and pigeon tissue were based upon its activating effect. Avitaminous tissue was very low in both the monophosphate and the pyrophosphate, but feeding the monophosphate brought about a rapid accumulation of the pyrophosphate in liver and brain.

*Animal physiology.*—A relatively new and striking viewpoint concerning the relation of vitamin-B<sub>1</sub> deficiency to nerve lesions was presented by Engel & Phillips from the Wisconsin laboratory (35).

The administration of  $\beta$ -carotene or percomorph oil together with riboflavin removed all evidence of pathology in the peripheral nerves of vitamin B<sub>1</sub> deficient chicks or chicks fed an adequate ration at a restricted level of intake. Neuropathology was seldom observed in a rat on a vitamin B<sub>1</sub> deficient but otherwise adequate diet. Recovery from vitamin B<sub>1</sub> deficiency produced a marked histologic reaction in the liver. The nerve degeneration encountered in human B<sub>1</sub> deficiency (beriberi) is probably due to a lack of dietary factors other than vitamin B<sub>1</sub>.

The work of Engel & Phillips points strongly toward a re-interpretation of the usual nerve lesions that occur on B<sub>1</sub>-deficient diets. Most of the B<sub>1</sub> deficiencies studied heretofore have been complicated by inanition and by deficiencies of other factors, particularly flavin and vitamin A. The striking response to B<sub>1</sub> therapy is apparently in large part due to improved appetite, assimilation, and general physiological changes not specifically related to the nerve lesions in a primary sense.

In agreement with the observations of Engel & Phillips, from a

chemical point of view, Lipschitz, Potter & Elvehjem (36) made a number of important observations on the close relation between inanition effects and vitamin-B<sub>1</sub> deficiency. They conclude that polyneuritis is a disease in which there is a general disturbance of carbohydrate metabolism rather than a condition that is characterized primarily by lesions in the nervous system. Liver and kidney tissue from both fasting and B<sub>1</sub>-deficient birds showed a lowered capacity to remove pyruvate. The brain tissue was disturbed similarly (low pyruvate removal) in B<sub>1</sub> deficiency but not from inanition alone. The ability of liver tissue to remove pyruvate was restored in both fasting and polyneuritic birds by oral administration of glucose. In the latter case (polyneuritis + glucose) there was glycogen deposition, but the livers from such birds did not regain a normal capacity to remove pyruvate. The authors emphasized the relation of pyruvate metabolism to the metabolism of other substances present in the tissues.

The close relation of vitamin-B<sub>1</sub>-deficiency symptoms to inanition was also indicated in a paper by Parade (37), who observed the importance of increased appetite and metabolism in parallel with recovery from bradycardia and subnormal body temperature. Thyroid feeding aided in alleviating the symptoms of B<sub>1</sub> deficiency, leading the author to suggest a "synergistic" relation of thiamin and thyroxin, rather than an "antagonism," as suggested by Drill (38). The latter observed that thiamin injection (500 International units per day) and B-complex feeding (5 per cent yeast concentrate) offset in large part the injury (weight loss) induced by thyroid feeding.

The regularity of onset of neuromuscular symptoms in rats was found by Schrader & Prickett (39) to be greatest (90 per cent) when the animals received a diet in which the nonprotein caloric intake was one half coconut fat and one half sucrose or corn starch.

The relation of thiamin, riboflavin and rice polish concentrate to fat metabolism in rats was investigated by McHenry & Gavin (40). The authors pointed out that casein, also, constituted an important variable in such experiments, and emphasized the fact that although body weight was roughly proportional to food intake on their experimental diets, the storage of body fat was not directly related in such a simple manner. It appears probable that fat synthesis and mobilization or distribution in the body is markedly conditioned by the intake of B<sub>1</sub>, B<sub>6</sub>, riboflavin, choline, and quality of casein.

The clinical value of thiamin for the alleviation of polyneuritis associated with alcoholism, particularly when associated with pellagra,

was emphasized by Spies & Aring (41), Goodhart & Jolliffe (42), Alsted & Lunn (43), Price (44), and Strauss (45). In their discussion of beriberi in alcohol addicts, Goodhart & Jolliffe stated that "cardiovascular disturbances of this nature are seen in approximately one third of the alcohol addicts admitted to this service (Bellevue Hospital, N.Y.) who show vitamin-B<sub>1</sub> deficiency in the form of peripheral neuritis."

Harris, Leong & Ungley (46) observed that normal, well-nourished, resting persons excreted 10 to 20 I.U. (International units) of thiamin per day. When given a test dose of 350 I.U., they excreted 30 or more I.U. They considered that resting excretion levels below 10 I.U., and levels below 15 I.U. after a test dose, indicated a sub-normal state of nutrition. Patients with nutritional polyneuritis showed daily excretion levels of 3.5 I.U., or lower, and a test dose excretion of about 2 I.U. Goudsmit & Westenbrink (47) found that men on a well-balanced diet excreted about 230 µg. per day, and women excreted about 120 µg. Women on a much poorer diet excreted about 40 µg. When given 5 mg. of vitamin orally, the above three groups excreted 560, 460, and 260 µg. respectively. These values appear to be very high in comparison with those of Harris *et al.*, and agree more nearly with those of Ritsert (48), who reported an average fecal excretion of 180 µg. per day on a normal diet.

A characteristic rise in the pyruvate content of blood, urine, and spinal fluid of patients with beriberi was observed by Platt (49). An intravenous injection of 5 mg. of vitamin restored the values to normal in ten to fifteen hours. Direct isolation of pyruvate as the 2,4-dinitrophenylhydrazone (1.5 mg.) from polyneuritic pigeon blood was reported by Shindo (50). The same method furnished 2 mg. of hydrazone from 150 gm. of human blood, taken from a patient with beriberi. After feeding *l*-phenylalanine, Closs & Folling (51) were able to detect very small amounts of phenylpyruvate in the blood of thiamin-deficient rats, but the quantity was too small to appear to be significant.

Feeding quantities of thiamin ranging from 15 to 515 µg. per day to rats resulted in a urinary excretion of 25 to 35 per cent and fecal loss of 20 to 30 per cent, in the experiments of Light and associates (52). The remainder was unaccounted for. "Saturation" of the tissues was established by feeding 50 to 65 µg. per day. When larger doses were fed after "saturation" there was an immediate increase in the urinary excretion, but when a state of deficiency existed, there

was a marked lag in the excretion rise. Subcutaneous injections of 200 to 500  $\mu\text{g.}$  into "saturated" rats was followed by urinary excretion of 75 to 85 per cent of the initial quantity. Only 50 per cent excretion followed injections of 2,000  $\mu\text{g.}$  into 10 kg. dogs.

Two papers from Scheunert's laboratory (53) showed clearly that there is no essential antagonism between vitamins A and B<sub>1</sub>.

Working with dogs, Dann & Cowgill (54) found that diarrhea did not affect the basic requirement of thiamin when supplied by injection, but when the vitamin was fed by mouth there was an additional requirement of 18 to 82 per cent, depending upon specific conditions of absorption.

In a long-time experiment by Drummond and associates (55), it was found that 483 rats on an adequate diet, compared to 556 on a diet that was suboptimal in vitamin-B<sub>1</sub> content, showed longer duration of life, better reproduction, and fewer gastrointestinal lesions (especially ulcerations).

The problem of providing an adequate diet for nursery school children, particularly with reference to vitamin B<sub>1</sub>, was considered by Robb, Vahlteich & Rose (56), with the conclusion that the following items should be included: milk 40, cereals (at least half should be whole-grains) 18 to 20, fruits and vegetables 16 to 20 per cent of the total caloric value, supplemented by 90 to 120 cc. of orange juice or its equivalent per day. In a well-controlled experiment with infants, Macy *et al.* (57) found that increasing the vitamin-B<sub>1</sub> content of a standardized diet by 30 to 50 per cent resulted in a distinctly more regular growth rate. Marked benefits in terms of the general growth and well-being of children as a result of special vitamin B<sub>1</sub> supplements were also recorded by Borsook *et al.* (58) and Schlutz *et al.* (59). In a study of the vitamin B<sub>1</sub> content of human milk, by the thiochrome method, Neuweiler (60) found values ranging from a trace to 13  $\mu\text{g.}$  per 100 cc., compared to 24 to 57  $\mu\text{g.}$  per 100 cc. in cow milk. Injection of the vitamin or feeding a yeast concentrate resulted in rises to the normal level. A review of the physiology and human requirement of vitamin B<sub>1</sub> was prepared by Cowgill (61), and Vedder has reviewed the general pathology of vitamin-B<sub>1</sub> deficiency (62).

In relation to comparative physiology, it is interesting to note Lwoff's observation (63) that the protozoan, *Acanthamoeba castellanii*, could synthesize thiamin from the pyrimidine and thiazole units. There appeared to be some capacity for synthesizing the thiazole

structure, but an intake of the corresponding pyrimidine unit was essential.

*Plant physiology.*—It has long been recognized that many of the lower plants such as bacteria, molds and yeasts can synthesize the entire B-group of vitamins, but considerable progress has been made recently in relation to the stimulating effect of the preformed vitamins and their respective structural parts. The special adaptations of plants have been studied also. Robbins (64) observed that a number of saprophytic fungi grew well in a nutrient medium essentially devoid of thiamin, but pathogenic fungi did not grow well unless the vitamin was supplied. The growth of *Rhizopus nigricans* was easily inhibited by thiamin. *Phycomyces nitens* grew well when the 5-bromo-methyl pyrimidine unit of vitamin B<sub>1</sub> and the thiazole unit were supplied, but either unit alone was inadequate. Six fungi grew well when the pyrimidine unit was supplied, with or without the thiazole unit, but could not grow when the thiazole unit was supplied alone.

Hills (65) observed that *Staph. aureus* required either thiamin or the pyrimidine unit plus the thiazole unit for normal growth and for the metabolism of pyruvate and lactate. In the absence of thiamin, pyruvate tended to accumulate and blocked the oxidation of lactate, both aerobically and anaerobically. Wood, Anderson & Werkman (66) found that the propionic acid bacteria responded to thiamin as a growth stimulant, but strains could be "trained" to grow well without it. Nilsson *et al.* found that an amyl alcohol-soluble factor from yeast was necessary to supplement thiamin for the growth of *Bacterium radiculicola* (67).

Bonner and associates studied the effect of vitamin B<sub>1</sub> and its two unit parts upon the growth of higher plants (68). Pea roots that had been cultured to free them from residual vitamin B<sub>1</sub> were markedly stimulated in their growth by minute amounts of thiamin, or equally, by the equivalent quantities of both of the unit parts. Either the pyrimidine or the thiazole alone were without effect ( $10^{-5}$  to  $10^{-11}$  M). Considerable evidence was obtained to show that synthesis of the vitamin occurred chiefly or entirely in the green leaves, as a result of photosynthesis, and was transported thence to the roots. Roots that had been stimulated or initiated by treatment with auxin were also sensitive to further stimulus by thiamin.



## RIBOFLAVIN

*Occurrence.*—An excellent review of the occurrence of riboflavin and its requirement by man and animals was prepared by Sherman & Lanford (1). The human requirement was estimated to be about 20 units (Sherman-Bourquin) per 100 calories. From a careful study of bioassays, including the use of pure synthetic riboflavin, Bessey (2) concluded that 2.0 to 2.5  $\mu\text{g.}$  of the pure compound were equivalent to one Sherman-Bourquin unit.

Whitnah, Kunerth & Kramer (3) published a report of extensive studies of composition of cow milk in relation to breed, ration, lactation period, and season. High or low riboflavin values corresponded roughly with variation in fat content, but could not be correlated with any other ingredient of the milk. Neither was there any regular correlation between riboflavin content and stage of lactation (fifteen days to ten months). The average values for flavin content (parts per million) during the spring months were: Ayrshires 1.17, Holsteins 1.37, Guernseys 1.53, and Jerseys 1.73.

Two papers served to emphasize the importance of riboflavin conjugation with milk protein. Ball (4) observed that xanthine oxidase, concentrated to 500 times the activity of whole milk, possessed the properties of a riboflavin-protein from which the prosthetic group could be separated by acid or alcohol. Corran & Green (5) isolated approximately 1 gm. of riboflavin-protein from 30 l. of milk and observed that it could serve as a catalyst for the oxidation of coenzyme I through such carrier agencies as methylene blue.

The riboflavin content of meats was studied by Darby & Day (6). The values found, based upon 1 Sherman-Bourquin unit = 2.5  $\mu\text{g.}$ , were: bacon 0.9, beef brisket 1.9, cured ham 2.0, lamb chops 2.8, fresh ham 3.0, and pork liver 23.0  $\mu\text{g.}$  per gm. The cataract-preventing capacity of the meats was proportional to their riboflavin content.

Carlsson & Sherman (7) measured the riboflavin content of tissues from rats that had received a constant diet of one third dried whole milk and two thirds ground whole wheat. The approximate ratio per unit weight, with skeletal muscle as 1, was: brain and spleen 2 to 3, heart 5, kidney 10, liver 10 to 20.

*Rôle in tissue respiration.*—Euler & Bauer (8) have shown that flavin-enzyme serves as an essential unit in the reactions by which glycogen can serve as a hydrogen donor for the reduction of methy-

lene blue in the Thunberg system. Rabbit muscle or rat sarcoma extract, together with a carbon dioxide precipitate from yeast extract (containing Robison-ester dehydrogenase), flavin-enzyme,  $\text{Co}^+$ ,  $\text{Co}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$  and  $\text{PO}_4^{---}$  provided all the essential factors for a rapid reduction of methylene blue by glycogen. Vigorous phosphorylation constituted a characteristic part of the reaction, and this too was dependent upon the presence of riboflavin.

A high capacity to phosphorylate riboflavin *in vitro* was shown by a protein concentrate prepared as a dry powder from the intestinal walls of rats, cats, and pigs by Hubner & Verzář (9). The enzyme was poisoned by iodoacetate *in vitro* and also by feeding the inhibitor before excising the tissue.

Warburg *et al.* (10) reported the isolation of a riboflavin-containing dinucleotide from yeast that was identical with the nucleotide from liver and kidney, and could serve as the coenzyme of *d*-amino acid oxidase. Karrer and associates were not in full agreement with this view, however (11). They believed that the dehydrogenation of *d*-alanine was due to an additional factor in the nucleotide studied by Warburg *et al.* Straub (12) also considered the *d*-alanine oxidase to consist essentially of a riboflavin dinucleotide linked to a protein. Warburg & Christian (10) believe that there are at least five distinctly different "yellow enzymes" of the riboflavin-protein type.

The ability of riboflavin to sensitize ascorbic acid to photocatalytic oxidation was studied by Hopkins (13). Reduced glutathione served to block the catalytic effect of the flavin. Schutz & Theorell observed (14) that four different types of facultative anaërobes did not alter their yellow enzyme content when subjected to aërobic or anaërobic conditions. The authors suggest that the finding provides another argument against the view that the enzyme functions as a direct hydrogen donor to molecular oxygen.

Other papers of special interest in relation to oxidation-reduction changes are those by Booher (15) (a review of the chemical aspects of riboflavin), Dickens & McIlwain (16) (phenazine compounds as carriers in the hexose monophosphate system), Karrer *et al.* (17) (octahydroflavins), and Clark (18) (potential energies of biological oxidation-reduction systems).

*Physiology and pathology.*—An excellent account of the histopathology of riboflavin deficiency in the chick was given by Phillips & Engel (19). Two types of deficiency symptoms were observed: (a) an acute paralysis, characterized as neuromalacia, and (b) a

slower, "curled toe" paralysis, reported earlier by Norris *et al.* Both types of deficiency could be prevented by adding pure riboflavin to the experimental diet. The main peripheral nerve trunks were especially involved, and characteristic changes were observed in the myelin. Stokstad & Manning observed similar phenomena in relation to the two types of deficiency (20). "Curled toe" paralysis was induced only when there was a delayed, partial deficiency. Jukes (21) found that the riboflavin requirement for poults was about the same as for small chicks, per unit weight of ration. He also reported that liver contained a growth factor in addition to riboflavin and the concentrated filtrates from rice bran, whey or liver. Lepkovsky & Jukes made the interesting observation that turkey poults developed a typical dermatitis as a result of riboflavin deficiency, even though an abundance of the "filtrate factor" was supplied (22). The lesions were readily cured by feeding riboflavin. Chicks did not show comparable lesions when grown on the diet, but did show slow growth, diarrhea and emaciation. The dermatitis of rats on the diet was distinct from the dermatitis of B<sub>6</sub> deficiency. Lepkovsky *et al.* also observed (23) an excess of fat in the livers of riboflavin-deficient birds, and a close relation between the riboflavin intake of hens and the hatchability of their eggs.

From a study of riboflavin deficiency in dogs, Sebrell & Onstott (24) reported that the following symptoms could be prevented by supplying the pure vitamin: bradycardia, cardiac arrhythmia, yellow mottling of the liver, degenerative changes in the central nervous system, collapse and coma.

The riboflavin content of casein was shown by Day & Darby (25) to account for the occasional failure of supposedly deficient rats to develop cataract. Mitchell & Cook reported (26) that riboflavin, thiamin and ascorbic acid were without value in preventing cataract that had been induced in rats by galactose feeding. Kuhn (27) reported that a thousand times the physiological requirement of riboflavin did not cause injury—the injury claimed earlier being due to the solvent used (N-methylacetamide). Demole (28) did not observe any injury from doses as large as five thousand times the protective level.

Riboflavin also serves as a growth stimulant for the propionic acid (29) and luminous (30) bacteria. Four different strains of *Micobacterium tuberculosis* were found to produce 0.5 to 2.86 µg. of riboflavin per cc. (31).

A good review of recent progress in the physiology and pathology of riboflavin was published early in the year by Hogan (32).

### NICOTINIC ACID (AND AMIDE)

An excellent illustration of the rapidity with which the results of a pure research on experimental animals may be used for the betterment of human health has been provided by the sequence of events following the discovery of the vitamin nature of nicotinic acid. A brief announcement by Elvehjem and associates of the fact that nicotinic acid (or its amide) had been identified as the anti-black tongue factor in liver extract, and that the same substance, by analogy, might cure pellagra, appeared in September, 1937 (1). Before the end of the year Spies' report (5) and a number of later papers from different medical centers carried accounts of the cure of human pellagra with the pure compound.

During 1938 there has been rapid progress in the study of nicotinic acid in relation to (a) human pellagra, (b) animal nutrition, (c) intermediate metabolism, and (d) plant growth.

*Identification as a vitamin.*—The first detailed paper by Elvehjem, Madden, Strong & Woolley (2) gave an account of the procedure used for the isolation of nicotinic acid and its amide, together with a discussion of the quantity of the acid in liver extracts (25 mg. per 100 gm.) and the dosage required to protect dogs (0.5 to 1.5 mg. per day). A second paper by the same authors gave an account of the relative black tongue-curative effects of a number of compounds related to nicotinic acid in molecular structure (3). Curative effects were observed only from compounds that would be expected, on a chemical basis, to be converted to nicotinic acid by oxidation or hydrolysis in the animal body. Ethyl nicotinate, nicotinic acid N-methyl amide, nicotinic acid N-diethyl amide, nicotinamide glucosidiodide and nicotinuric acid were all active when given orally. Trigonelline, nicotinamide methochloride, pyridine, pyridine- $\beta$ -sulfonic acid, 6-methylnicotinic acid,  $\beta$ -acetylpyridine, quinolinic acid, picolinic acid, isonicotinic acid, nipecotic acid and nicotinonitrile were inactive at levels three to four times higher than the curative level of nicotinic acid.  $\beta$ -Picoline showed a degree of activity between that of the above two groups. In agreement with the above findings, a report from another laboratory that  $\beta$ -aminopyridine could cure black tongue was found to be in error—the compound has no activity

in curing black tongue or in stimulating the growth of *Staph. aureus* (4).

Spies and associates (5) have been particularly active in the study of clinical pellagra. A curative dosage of one half gram daily was considered to be a safe and very effective level. Porphyrinuria (characteristic of pellagra and also of some nonpellagic conditions) was promptly cured when the acid was fed. The appearance of nicotinic acid in the urine, as measured by the color reaction with 2,4-dinitrophenylchloride, appeared to offer a basis for estimating the state of nutrition of an individual and a valuable means of detecting the prepellagic state. The following statement by Matthews, based upon experience with thirteen "classic cases" of pellagra, is apparently typical of clinical experience with nicotinic acid:

The most striking as well as the most gratifying observations in my experience were the rapid healing of lesions in the alimentary tract with the development of an excellent appetite and gastrointestinal function, and the spectacular disappearance of mental symptoms [6].

In a study of the dog's requirement for nicotinic acid, Sebrell and associates (7) found that the total requirement to effect a cure ranged from 20 to 60 mg. for 6 to 8 kg. animals. Semiweekly feedings of 3 mg. were barely sufficient to prevent the occurrence of black tongue through a six months period. Margolis, Margolis & Smith (8) reported that 0.5 mg. per kg. per day was the minimum curative dosage. Higher levels of feeding gave almost the same response as 0.5 mg. When the daily level was only 0.2 mg., the curative response was markedly delayed, about five to eight days being required to cure the mouth lesions and to permit a slow resumption of growth. There was no evidence of decreased activity after autoclaving for six hours.

Helmer & Fouts (9) showed that the conventional diets used for the production of black tongue are deficient in both riboflavin and filtrate factor, and observed only a slight increase in rat growth when riboflavin was added to such diets. It is suggested accordingly by these authors and others (10) that general dietary improvement should be provided for pellagrins in addition to effecting a specific cure with nicotinic acid. It was somewhat surprising to find that an intravenous injection of 50 mg. of cozymase (diphosphopyridine nucleotide) did not exert a significant effect upon the course of black tongue (11). Euler, Malmberg, Robeznieks & Schlenk (12) observed that crude cozymase from yeast, even after treatment with strong

alkali, served as a better supplement than pure nicotinic acid amide, to riboflavin and thiamin, in the diet used.

Dann & Subbarow (13) showed that nicotinic acid was distinct from both the chick and the rat antidermatitis factors.

Independently, Mickelsen, Waisman & Elvehjem (14) observed that nicotinic acid had no effect upon chick dermatitis. Neither did the acid or its amide influence the growth of chicks when they were fed the Goldberger pellagra-producing ration. The chick antidermatitis factor from liver extract was also clearly differentiated from factor W, riboflavin, B<sub>1</sub>, and B<sub>6</sub>.

Day reported that nicotinic acid would not protect monkeys from nutritional cytopenia (15) and Harris (16) showed clearly that a condition described as "monkey pellagra" could be promptly cured by feeding 5 mg. of the acid per day to 2 kg. animals or proportionate dosages to larger animals. Day suggested that the nutritional cytopenia resulted from a deficiency in a new factor (M).

Chick and associates (17) found that pigs could be grown satisfactorily on a diet of corn 77.5, purified casein 6.5, ground peas 10.5, cod-liver oil 3, and salt mixture 2.5 parts, when a supplement of 60 mg. per day of nicotinic acid was provided. The addition of riboflavin in place of nicotinic acid did not improve the diet for pigs. Rats were found to grow rapidly for long periods when given the basal pig diet, providing further evidence that if the rat requires nicotinic acid at all, the requirement is relatively low.

An independent method for the isolation of nicotinic acid from liver was described by Ackermann & Fuchs (18).

*Methods of analysis.*—The problem of determining nicotinic acid and its amide in solid tissues, blood, and urine has been studied in a number of laboratories with reasonably satisfactory results (19). The reaction with 2,4-dinitrophenylchloride has been most widely used, although tests based upon the growth of *Shigella paradysenteriae* offered promise of quantitative interpretation (20).

*Tissue function.*—In view of the fact that nicotinic acid amide had been discovered as the part of coenzymes I and II that undergoes reversible oxidation and reduction, before the acid had been identified as a vitamin, it has been assumed that the coenzyme function represents the main tissue activity of the vitamin. The possibility of additional functions cannot be excluded on the basis of its being an essential part of the coenzymes, however. Since tissue oxidation-reduction reactions have been treated in a separate chapter, only a

very brief review of the rôle of nicotinic acid amide will be given here.

Euler, Schlenk & Forsman (21) described the carrier function of coenzyme II in Jensen sarcoma in catalyzing the oxidation of Robison's ester as a substrate in the Thunberg technique. Hexose-diphosphate and the dehydrogenation product of Robison's ester (phosphohexonic acid) served as active hydrogen donors in a series of reactions that accomplished the oxidation of glycogen, together with intermediate phosphorylation. Conversion of coenzyme I to coenzyme II by a yeast press-juice was described by Euler *et al.* (22).

Evidence in support of Ostern's theory that cozymase breaks down during its oxidation cycle to form adenosinediphosphate and dihydropyridinenucleotide, to be followed by recombination of the nucleotide and adenosine, was given by Lennerstrand (23). Modified methods for the preparation of purified cozymase and dihydrocozymase from yeast were given by Ohlmeyer (24).

A new respiratory catalyst that is much more active than riboflavin-protein in the oxidation of coenzymes I and II was separated from rabbit muscle, pig heart, yeast and several bacteria by Dewan & Green (25). The "coenzyme factor" catalyzes the transfer of two hydrogens from the reduced coenzymes to such hydrogen acceptors as cytochromes a and b, riboflavin, and adrenochrome. The preparation had the general properties of a protein, but little progress was made in determining its chemical nature. It appears probable, however, that this new catalyst is the major specific agent that acts on the reduced form of nicotinic acid amide in cellular respiration.

A closely related finding by Euler & Hasse (26) is also of major interest. They obtained an active protein extract from rat muscle, named "diaphorase," that served as an active catalyst for the transfer of hydrogen from dihydrocozymase to molecular oxygen or methylene blue. The enzyme was also present in brain, heart, kidney, intestinal wall, hypophysis, ovary, placenta and thyroid. It did not act upon coenzyme II, however.

An *in vivo* regulation of cathepsin activity by dihydrocozymase is indicated by the work of Karrer & Straus (27).

Schlenk, Hellstrom & Euler (28) studied the function of the adenylic acid portion of the cozymase unit, and observed that when the coenzyme was treated with nitrous acid, forming desaminocozymase by removal of the amino group from the purine base, there was a two-thirds loss in the hydrogen-carrier activity of the nicotinic acid

amide group. Although the combining power of the coenzyme unit with the protein (apoenzyme) was changed by the deamidation, the authors did not think that the change was a regulatory one in relation to phosphorylation. When the resulting inosinic acid unit was combined with the nicotinic acid amide nucleotide unit and apoenzyme again, there was no evidence of phosphorylation catalysis.

From a study of oxidation-reduction reactions in muscle extract, Needham & Lu (29) concluded that the first step, oxidation of triosephosphate by coenzyme, was accompanied by esterification, but that the next step, oxidation of reduced coenzyme by pyruvate, is not accompanied by esterification. Adenylic acid was observed to have an activating effect upon the oxidation of triosephosphate, and an inhibiting effect upon the oxidation-reduction of glyceric aldehyde. Similarly, the oxidation of reduced coenzyme by acetaldehyde in yeast respiration was found by Meyerhof, Ohlmeyer & Mohle (30) not to be related directly to phosphorylation. When the resulting inosinic acid unit was combined with the nicotinic acid amide nucleotide unit and apoenzyme again, there was no evidence of phosphorylation catalysis. Isolation of the *d*-alanine oxidase coenzyme from horse kidney as the barium salt,  $C_{27}H_{31}O_{15}N_9P_2Ba$ , was announced by Warburg & Christian (31). The oxidation-reduction reactions of the pyridine-proteins were discussed in detail by Warburg, in a comprehensive review (32).

Evidence for the rôle of coenzymes I and II in the dehydrogenation of glutamic acid and reconversion of the ketoglutaric acid to glutamic acid was studied in detail by Euler, Adler, Gunther & Das (33). They believe that the glutamic acid-ketoglutaric acid system accounts for a major part of (a) the cellular respiration, and also for (b) the ability of the body to reconstruct amino acids through intermediate keto acids.

*Influence on plant growth.*—Koser, Dorfman & Saunders (34) found that the growth of dysentery bacilli was dependent upon the presence of one or more of the compounds shown by Elvehjem *et al.* to be effective in curing black tongue in dogs. The series of compounds that gave positive or negative results closely paralleled those tested in animals, and the results were strikingly similar. Only those that could readily give rise to nicotinic acid by hydrolysis or oxidation were effective.

Landy observed that nicotinic acid and nicotinyl glycine served as growth-essential factors for *Staph. aureus* (35). Positive effects were



also recorded for six species of lactic acid bacteria (36), *Proteus* (37), isolated pea roots (38), *Staph. aureus*, fungi and *Protista* (39).

A review of the work leading to the identification of nicotinic acid as the antipellagric factor was published by Sebrell (40).

### VITAMIN B<sub>6</sub>

Crystalline vitamin B<sub>6</sub> was obtained in a number of laboratories during the year, and there was good agreement concerning the composition and assay value of the products isolated. Rats were cured of dermatitis and resumed growth when fed 5 to 10 µg. of the hydrochloride per day.

György published a detailed procedure for the isolation of vitamin B<sub>6</sub> (1). In a later paper (2) he called attention to: (a) the higher requirement of the vitamin when the test animals were kept at a low temperature (40° F.); and (b) the possibility that chilblains may afford a clinical demonstration of vitamin B<sub>6</sub> deficiency.

Independent methods of isolation were published by Lepkovsky (3), Emerson *et al.* (4), Ichiba & Michi (5), Keresztesy & Stevens (6), and Kuhn & Wendt (7). The accepted formula for the hydrochloride is  $C_8H_{11}O_3N \cdot HCl$ . The compound (m.p. 204–6°) is not changed by treatment with strong acids, alkali, nitrous acid, or Fehling's solution, and shows strong absorption bands at 2925 and 3275 Å. at pH 4.5 (6). The acetate is biologically active and can be distilled *in vacuo* (7). Diazomethane forms a methyl ether which, in turn, gives a diacetate of the ether, m.p. 54°, formula  $C_{13}H_{17}O_5N$ . It is concluded (7) that the molecule contains two alcohol groups, one phenolic group and a tertiary cyclic nitrogen. Kuhn observed that the vitamin was nondialyzable from aqueous yeast extract, which may indicate its presence in combination with protein.

Lunde & Kringstad (8) found fresh cod liver to be rich in vitamin B<sub>6</sub>, 0.25 gm. per day being adequate for growth and rapid cure of severe dermatitis. Cod roe was also a good source (0.5 gm. per day) and 2 gm. per day of fresh cod meat or canned sardines provided a sufficient quantity for growth and rapid cure of dermatitis. *d*- or *d,l*-Leucine was found to have no effect upon the development of dermatitis.

Fouts, Helmer, Lepkovsky & Jukes (9) observed the development of microcytic hypochromic anemia in puppies that were fed a diet containing adequate quantities of all the other recognized factors

except vitamin B<sub>6</sub>. When vitamin B<sub>6</sub> in the form of rice polish extract was fed as a supplement to the diet, the anemia was cured more rapidly (two to three days) than is common for pernicious anemia or nutritional anemia (five to ten days).

Birch (10) found that the unsaturated fatty acids of maize oil were effective in alleviating the symptoms of vitamin-B<sub>6</sub> deficiency, and suggested that this finding was related to the observations of Burr & Burr concerning essential fatty acids, and perhaps also to the fat-soluble antidermatitis factor reported by Hogan & Richardson. He could find no evidence to indicate that the vitamin might occur in combination with lipids. Instead, the evidence pointed toward a functional relationship between the unsaturated fatty acids and the vitamin. Further evidence in support of such a view was provided by Halliday (11) who observed an accumulation of fatty acids in vitamin-B<sub>6</sub>-deficient animals. Feeding choline caused a decrease in the excessive lipid storage, but did not afford complete protection. Quackenbush & Steenbock (12) found that rats were protected from acrodynia and continued in good health when maintained on a B<sub>6</sub>-deficient diet that was supplemented with unsaturated fatty acids, either as natural oils or as 10 mg. per day of ethyl linoleic ester. Salmon (13) also observed a relation between B<sub>6</sub> and fat metabolism.

The rôle of vitamin B<sub>6</sub> as a growth factor for bacteria (lactic acid formers) is indicated by the positive findings of Moller (14).

## VITAMIN-B COMPLEX

### FACTORS OTHER THAN B<sub>1</sub>, B<sub>6</sub>, RIBOFLAVIN AND NICOTINIC ACID

The identity of several "vitamin-B" factors besides thiamin, riboflavin, nicotinic acid, and B<sub>6</sub> that have been recorded in the literature remains somewhat problematical. Choline, too, may well be considered as a member of such a group. The following factors may each represent more than one substance, fed as a mixture, or the list may include the same substance under two or more names, due to different techniques of study in different laboratories. The value of studying the nutritional requirements of different animals, and the great advantage that is afforded by the use of pure compounds in animal nutrition have both been illustrated by the progress being made in attempting to identify the great number of factors in the old "vitamin-B" complex.

*Grass juice factor.*—Kohler, Elvehjem & Hart (1) have continued the study of a thermolabile, water-soluble material from green grass that is essential for the nutrition of guinea pigs. Apparently the factor is not essential for rat growth, but it may account in part for the better growth of rats when fed summer milk in comparison with winter milk. As a matter of fact, very little is known of the qualitative or quantitative requirement of the guinea pig for water-soluble factors other than vitamin C, due in large part to their failure to grow well on purified diets that are comparable to those used for rats.

*Lactation factors (vitamins  $L_1$  and  $L_2$ ).*—The Japanese workers have continued to present evidence for the existence of two water-soluble lactation factors (2) that may be concentrated from beef liver ( $L_1$ ) and baker's yeast ( $L_2$ ). The basal diet, consisting of polished rice powder 75 gm., fish protein 10 gm., butter 10 gm., McCollum's salt mixture 5 gm., supplemented with acid earth adsorbate (vitamin B), was adequate for good growth (rats) and normal gestation but did not become adequate for lactation unless approximately 50 mg. of  $L_1$  and 15 mg. of  $L_2$  were supplied daily. Larger amounts of either  $L_1$  or  $L_2$  could not compensate for the other factor. When lactation had been established with the first litter, much smaller quantities of  $L_1$  and  $L_2$  were required to induce lactation for a second litter. The authors suggest that the lactation factors are of special importance in the maturation of the lactation tissues, rather than for supplying a material that is essential to continued milk formation.

In view of the often-observed failure of lactation and suckling when diets are marginal in supplying known nutrients, however, the observations of Folley and associates (3) are of special interest in relation to the claims for lactation-essential vitamins. The investigators at Reading could not verify the findings of Nakahara and associates, although their experimental procedure was very similar to that employed by the Japanese workers. Differences in the vitamin content of the fish meal used in the diets might account for the variance in results, however.

*Muscular dystrophy.*—Morgulis *et al.* (4) observed in rabbits a type of muscular dystrophy that was due to a deficiency of a water-soluble factor, and therefore distinct from vitamin E, shown by Mattill & Olcott (5) to be essential for the prevention of muscular dystrophy. Mattill suggested, however, that oxidative destruction of vitamin E by rancid fats, as in cod-liver oil, might give rise to a misinterpretation of the need for a water-soluble factor (5).

*Additional rat factors.*—Euler and associates (6) observed a supplementary effect upon rat growth when cozymase and yeast filtrate were fed simultaneously. Earlier tests for the effect of nicotinic acid upon rat growth had left the problem unsolved, but this finding in relation to cozymase appears to offer a means of identifying one of the individual units in cozymase as a rat-growth factor.

Lunde & Kringstad (7) observed that 20 µg. per day of a water-soluble fraction from fish liver served to permit growth in rats, and to protect them from a peculiar type of hair discoloration. The latter was characterized by the development of a reddish-brown discoloration about the neck and forehead, and with pied rats, the black hair showed an additional tendency to turn gray. Morgan *et al.* (8) observed a close correlation between filtrate factor intake and graying of hair in black rats. They also emphasized the advantage in using sucrose (compared to lactose) for B-complex assay diets, to avoid intestinal synthesis of the vitamins.

*Chick factors.*—The chick antidermatitis factor described earlier by Elvehjem and associates was concentrated and characterized in greater detail by the Wisconsin laboratory (9). The vitamin is acidic and soluble in ethyl acetate, dioxane, and glacial acetic acid, but is insoluble in the common fat solvents. After acetylation it is soluble in chloroform, ether and benzene, and the acetyl derivative can be distilled at 100°, 10<sup>-5</sup> mm. Physiological activity was restored by hydrolysis of the acetate. Nitrous acid did not destroy the activity, indicating the presence of a hydroxyl group and the absence of a primary amino group.

Stokstad & Manning (10) suggested the name Factor U for a vitamin that is essential for chick growth and apparently different from thiamin, riboflavin, filtrate factor, B<sub>4</sub>, or the anti-encephalomalacia factor. Characteristics of the product were as follows: (a) found in alfalfa-leaf meal, yeast, wheat bran, middlings, and to a lesser extent in corn; (b) soluble in 50 per cent methyl alcohol, insoluble in ether, acetone and isopropyl alcohol; (c) adsorbed on fuller's earth and charcoal; (d) destroyed in alfalfa by autoclaving five hours at 120°, but not destroyed in yeast by autoclaving or refluxing for thirty minutes at pH 1.7 or 11.0.

There has been continued disagreement between the findings in different laboratories relative to the anti-gizzard erosion factor for chicks. The Wisconsin group has obtained consistent protection and improved growth by feeding chondroitin and chondroitin-containing

products (11, 12), but they could find no evidence in support of the finding by Almquist that the active material might be of lipid nature or might influence growth. Both laboratories were in agreement concerning the benefit of feeding bile salts. There was evidence that glucuronic acid might be the active unit in chondroitin.

The complexity of chick requirements is further illustrated by the findings of Jukes & Babcock (13) in studying the growth and anti-paralysis value of alfalfa meal. The same protective action was provided by a fat-soluble fraction from soybean oil and a water-soluble fraction from alfalfa meal.

Good reviews concerning the "vitamin-B complex" were published during the year by Nelson (14) and McCay (15).

The status of vitamin B<sub>6</sub> and B<sub>12</sub> remains unsettled.

### VITAMIN C (ASCORBIC ACID)

The number of papers dealing with vitamin C has been very great, due in large part to the ease of determining its approximate concentration in tissues, the baffling uncertainty regarding its various apparent functions, the rapidity with which it can be oxidized by air or common laboratory reagents, and an increasing recognition of its general physiological importance in both animals and plants.

*Type-reactions and properties.*—Absorption spectra of ascorbic acid and analogous compounds were studied by Mohler & Lohr (1), with special reference to the effect of cyanide in causing a shift of the bands toward the red. The absorption maxima in dilute aqueous solutions were: *l*-ascorbic acid 252 m $\mu$ , reductic acid 267 m $\mu$ , hydroxy-tetronic acid 246 m $\mu$ , and mesityl oxide 244 m $\mu$ .

Six-membered ring compounds analogous to ascorbic acid were prepared by Haworth *et al.* from ketogluconic acid and from fructose (2). A number of syntheses related to ascorbic acid were also carried out by Reichstein and associates. *d*-Ascorbic acid was prepared from *d*-sorbose, using the diacetone intermediate and permanganate oxidation, as carried out previously for *l*-ascorbic acid (3). 6-Desoxy-*l*-ascorbic acid was prepared from 2,3-monoacetone-*l*-sorbo-methylose in a similar manner (4). The desoxyascorbic acid proved to have about one third of the antiscorbutic activity of *l*-ascorbic acid (5)—the highest antiscorbutic activity of any compound other than *l*-ascorbic acid thus far studied. Methyl-2-ketogluconate (6) was found to protect against scurvy when fed at a level of 30 mg. per day.

The reaction of dehydroascorbic acid upon a series of  $\alpha$ -amino acids, resulting in their degradation to aldehydes with one less carbon atom, ammonia, and carbon dioxide, was studied by Abderhalden (7). Dimedon derivatives of the aldehydes were prepared from  $\alpha$ -aminobutyric acid, valine, norvaline, leucine, isoleucine, *norleucine*, *allo*-isoleucine and phenylalanine. Preliminary evidence of serum protein combinations with ascorbate was reported by Jonnard & Ruskin (8). The observations of Rudolph (9) concerning ascorbic acid combinations with proteins and amino acids, causing stabilization of the acid, can possibly be explained in large part by the effect of the added substances upon copper ion. Parrod (10) reported an interesting type-reaction of ascorbic acid, when it is oxidized in the presence of ammonia or primary amines. A series of oxamides,  $(\text{CONH}_2)_2$ , and N-substituted analogues, were formed and isolated in fairly good yields.

Largely on the basis of discrepancies between the loss in indophenol titration value and oxygen uptake by ascorbic acid, Bezssonoff & Woloszyn (11) postulated an intermediate oxidation step in which one oxygen atom removes one hydrogen atom from each of the enolic groups on C-3 of two molecules of ascorbic acid. A second oxygen atom then removes the two hydrogens from C-2, giving rise to dehydroascorbic acid. In view of the uncertainty concerning the nature and reactivity of the intermediate peroxide that may be formed, however, it is not necessary to interpret the oxygen-titration discrepancies on the above specific basis. Photochemical decomposition of ascorbic acid by ultraviolet light without the presence of oxygen or riboflavin was reported by Zilva (12). The general chemistry and physiology of the vitamin were summarized in an earlier review (13).

*Analytical methods.*—The direct titration of ascorbic acid by 2,6-dichlorophenolindophenol in acid solution has continued to be the most satisfactory method of analysis for general use with food products and fresh tissues. Precautions must be observed, however, in relation to the following points:

- a) the dye must be free from interfering impurities, carefully standardized, and used only in fresh solution;
- b) the extraction should be made with a strong acid, preferably 3 per cent metaphosphoric, accompanied by thorough grinding in such a manner that extraction is complete and oxidation does not occur before protection is provided by the acid;
- c) the titration must be carried out rapidly with agitation and should be stopped when the color persists for more than five to ten seconds;

- d) the possible presence of the reversibly oxidized form of the vitamin must be considered, and if present, provision must be made for quick reduction with hydrogen sulfide in a suitable pH range, followed by removal of the hydrogen sulfide without permitting reoxidation of the vitamin; and
- e) the method should not be used for analyzing products that contain significant amounts of interfering substances, such as malt, beer, and solutions containing ferrous ion, sulfides and thiosulfates.

Bessey published a review of the literature (14) and an excellent experimental paper (15) in which each step in the analytical procedure was carefully examined. The indophenol indicator was used, with 3 per cent metaphosphoric acid as extractant and a photoelectric colorimeter (Evelyn type) for the readings. The method permits analyses to be made within 3 per cent error in the presence of nominal amounts of suspended matter, such as glycogen and barium sulfate, coloring matter (as in beets, berries, and food dyes), dehydroascorbic acid, glutathione, and cysteine. Emphasis was placed upon the importance of buffering within the pH range 3.5 to 3.7, and upon the failure to find evidence for incomplete extraction with the acid used. Data were given to illustrate the applicability of the method to a great variety of plant and animal products, including urine.

The indophenol reagent and a photoelectric colorimeter for ascorbic acid determinations in urine were investigated also by Evelyn, Malloy & Rosen (16). The authors advise extrapolation of a dye-reduction-time curve to correct for the interference of other reducing substances in the urine. They reported that *low-titration* urines often showed the presence of two to ten times more nonvitamin reducing material than true ascorbic acid. Direct titrations were found to be valid for high excretion levels, however, so that for diagnostic purposes, the direct titration value would not generally be misleading. The removal of interfering material by precipitation with mercuric acetate, as suggested by Emmerie *et al.* (17), was found to be of little, if any, value. The addition of barium acetate for the removal of interfering substances was found to be beneficial in many cases. Their observations indicate that human excretion levels below 5 mg. per day are common, even on "normal" dietaries, but it is likely that their values were occasionally too low because of the failure of 5 per cent acetic acid to act as an adequate preservative for twenty-four-hour samples. Both Bessey and Evelyn agree that reduction of the dye by ascorbic acid is normally complete in less than five seconds.

The special problem of ascorbic acid determination in blood plasma and spinal fluid by means of indophenol and the photoelectric

colorimeter was studied by Mindlin & Butler (18). A time interval of thirty seconds was used, and the procedure was described for use in both macro- and micromanipulation. The question of whether cyanide should be added to blood serum samples was considered by Friedman *et al.* (19), who found that the step was not necessary. Further reports on the indophenol titration method for determining ascorbic acid in milk were published by Willberg (20) and Sharp (21).

The question of ascorbic acid occurrence in protein conjugates that resist acid extraction was considered by Thornton (22) and Reedman & McHenry (23). The former concluded that McHenry's claim for the existence of significant quantities of such conjugates was invalid and perhaps based upon incomplete extraction and incomplete protection from oxidation during extraction (in agreement with the experience of Tressler, Bessey, King, *et al.*). The risk of vitamin loss from tissue during the interval after death was emphasized in a paper by Peters & Martin (24).

Simplified and improved methods of dye standardization were described by Menaker & Guerrant (25), and Buck & Ritchie (26), based upon the use of a standard thiosulfate titration of the iodine liberated from potassium iodide by the oxidized form of the dye in acid solution. Lorenz & Arnold proposed the use of ferrous salts (27), very much as in Tillmann's early procedure. Methylene blue was again proposed by Policard *et al.* (28), who found it to be useful, but the authors were not certain of its specificity.

By using cadmium chloride to inhibit mercuric chloride oxidation during precipitations, and by introducing a number of steps to avoid interfering reactions, Kassell & Brand (29) developed a modified Folin-Lugg procedure for the analysis of high-sulfur urines and protein hydrolysates in which cystine, cysteine, sulphydryl compounds and ascorbic acid could be measured. The method is based essentially upon the reduction of phosphotungstic acid in the presence of cadmium and mercury salts, and recording the color developed in a Pulfrich photometer.

A slow-reacting, yellow-to-lilac oxidation indicator, cacothelin, was proposed by Rosenthaler (30) for the determination of ascorbic acid in mixtures with cysteine and glutathione, but practically no evidence was given for its general applicability to tissue analysis. Scudi & Ratish (31) found that reduction of an azo dye by ascorbic acid could be used as a basis for vitamin-C determination in pure solutions



in the presence of cysteine and in citrus juices. Hydroquinone and glucoreductones interfered, however. The units coupled with diazotized sulfanilamide and dimethylnaphthylamine, and the readings were made colorimetrically. Woker & Antener (32) suggested the decomposition of ascorbic acid to furfural, as suggested earlier by Roe, to be followed by the development of a color in the distillate by reaction with alkaloids or sterols. Stillman (33) studied the quantitative reaction between ascorbic acid and ceric ion, but did not apply the procedure for general analytical purposes. A preliminary paper by Espil & Genevois (34) pointed out the possibility of separating the osazone of dehydroascorbic acid and 2,4-dinitrophenylhydrazine and then titrating the resulting product with titanium trichloride—a reaction involving thirty hydrogen equivalents per mol. Fujita & Sakamoto (35) reported satisfactory analyses based upon direct spectrometric measurement, utilizing the absorption maximum at 240 m $\mu$ . They found good agreement between the increased indophenol titration value and the increased concentration of ascorbic acid, as measured by the absorption intensity after hydrogen sulfide reduction.

*Vitamin content of foods.*—It seems scarcely possible adequately to summarize the large amount of information that has been published relative to the vitamin content of foods, because its significance lies primarily in the detailed quantitative data given for individual products and processes. Even tabular summations of the data would require a disproportionate amount of space in a review of the present type. The studies are of major importance, however, and will be cited in a number of cases where extensive work has been done.

Tressler and associates have continued their comprehensive survey of the vitamin-C content of American vegetables (36). Special attention has been given to the effect of soil condition, varietal differences, harvesting practices, maturity and size, storage conditions, freezing methods, and cooking losses. Lunde and associates (37) have also continued their studies of the vitamin-C content of Norwegian foods, including a great variety of vegetables, fruits, algae and fish products. Both laboratories have supplemented their chemical titration data with biological assays whenever there seemed to be a critical need for such work. Olliver (38) has continued the study of English foods, with special attention to fruits and vegetables.

Scheunert (39) did not find major differences in the vitamin-C content of vegetables that could be attributed to the effects of different fertilizer practices. Papers dealing specifically with the vitamin-C

content of apples (40), potatoes (41), peas (42), milk (43), tomato juice (44), and East Indian vegetables (45), may also be of interest to many readers. Investigations of freezing and storage practices in relation to fruits and vegetables were reported by Plagge (46) and Fitzgerald & Fellers (47).

*Plant physiology.*—A specific stimulating effect of ascorbic acid on the growth of plant embryos was shown by the work of Bonner & Bonner (48). The varieties of peas showing a highly developed synthetic capacity were relatively less responsive to added vitamin C in the nutritive medium than those with a lower rate of synthesis. A dependence upon reserve food material for synthesis was also shown by Bonner, but the specific nature of the precursor was not identified or studied in detail.

Another important lead concerning the rôle of vitamin C in plants was found by Weier (49). He concluded from a study of the reducing material that accumulates in green leaves during photosynthesis that the reducing action often attributed to  $n\text{-}\Delta^2\text{-hexenal}$  was probably due to ascorbic acid. The independence of chlorophyll and vitamin C in the different structural parts of plants was emphasized by Mirimanoff (50). In a preliminary paper Rudra (51) reported that minute quantities of manganese added to seeds favored ascorbic acid synthesis during germination. A review of the literature, together with experimental evidence of the marked effect of light and a glucose supply upon ascorbic acid synthesis in plants, was published by Reid (103).

An increased growth rate in cultures of microorganisms as a result of adding ascorbic acid to the medium has been reported from a number of laboratories. Unfortunately most investigators have not carried their work far enough to differentiate the various factors involved, such as oxygen tension, reduction potential, pH, peroxide formation, rate of vitamin loss, or bios-type function. Kligler *et al.* attempted to analyze the various factors in relation to the growth-stimulating effect of ascorbic acid on *Clostridium welchii* (52) and *Cl. tetani* (53). They concluded that  $E_h$  was of much more critical importance than oxygen tension, and that ascorbic acid served as a detoxicating agent both during and after the growth of the organisms. Farber observed a marked stimulating effect of the vitamin on the growth of *Staph. aureus* (54). The relative rates of destruction of vitamin C in cultures of a great variety of organisms were studied by a number of investigators. In one case a correlation was observed

between rate of destruction and rate of fat oxidation (55). Berencsi & Illényi (56) reported the formation of relatively large amounts of indophenol-reducing material by *B. prodigiosus* in the presence of *d*-xylose, but there was no direct or biological proof that the product was ascorbic acid.

*Relation to specific plant enzymes.*—Contrary to many earlier reports, Ohlsson & Thorn (57) found that ascorbic acid did not exert a significant influence on the formation of  $\alpha$ -amylase in sprouting barley. A peculiar inhibiting effect of ascorbic acid + copper ion on extracted soybean phosphatase was observed by Giri (58). The inhibition was not due to copper, ascorbic acid, or dehydroascorbic acid alone, but the possibility of a peroxide effect was not eliminated. A number of sulfur compounds destroyed or blocked the inhibiting effect.

Several papers have dealt with the natural catalysts that are essential to the aërobic oxidation of ascorbic acid below pH 7.6. Kertesz (59) could not duplicate the findings of Hopkins & Morgan on the carrier rôle of ascorbic acid in promoting the aërobic oxidation of glutathione in cauliflower juice. Neither could he observe such an effect with cucumber juice. Crook & Hopkins (60) then reported that they could fully duplicate the earlier findings in the Cambridge laboratory. The presence of a catalyst that promoted reduction of dehydroascorbic acid by glutathione in cauliflower juice (and in cabbage) was again found, in addition to a catalyst for the oxidation of ascorbic acid. They found small amounts also in cucumber juice. The authors suggested that the failure of Kertesz to observe the phenomenon was due to not having fresh tissue from which to prepare a press-juice.

The field of protein-copper catalysis and substrate specificity has been studied actively. Shortly after Stotz, Harrer & King published evidence that the catalytic effect of plant juices on the oxidation of ascorbic acid was *in large part* due to copper-protein catalysis, Kubowitz reported that polyphenol oxidase from potato press-juice was a copper-protein complex. Silverblatt & King reported further evidence of the copper-protein nature of the catalytic agency responsible for vitamin-C oxidation in a number of plant juices (61). Kubowitz (62) reported observations on the reversible removal of copper from polyphenol oxidase and hemocyanin, and, in addition, studied the substrate specificity of the partially purified enzyme. The polyphenol oxidase did not serve as a catalyst for the oxidation of ascorbic acid,

hydroquinone, or resorcinol, but was active upon the following substrates: catechol, tyrosine, adrenaline, dihydroxycinnamic acid, pyrogallol, dopa, and protocatechuic acid. In view of the availability of tyrosine as a substrate for catechol oxidase, it is of interest to note that Dalton & Nelson (63) have isolated a crystalline tyrosinase from mushrooms that proved to be a copper-protein. It was catalytically active on both tyrosine and catechol.

Szent-Györgyi and associates (64) studied the mechanism by which copper serves as a catalyst in promoting the reduction of methylene blue by ascorbic acid. They also made a preliminary study of the copper content of cucumber oxidase, and found that the soluble ash was less active than the enzyme preparation, indicating the presence of other agencies or an activation of copper in the organic complex.

The nonspecificity of cucumber oxidase toward compounds related to ascorbic acid was emphasized in a paper by Snow & Zilva (65). *d*-Glucoascorbic, dihydroxymaleic and reductic acids were oxidized at a slower rate than *l*-ascorbic acid by molecular oxygen, in either dialyzed or undialyzed press-juice. After dialysis, however, the press-juice was inactive toward dihydroxymaleic acid, but active toward the other two substrates, indicating that the latter were oxidized by a different agency.

*Functions in the animal body.*—From a strictly chemical point of view, perhaps less information is available concerning the rôle of the vitamin in animal tissues than in plant tissues. The many functional relationships that have been established for the vitamin in the animal body are still largely empirical.

Giroud published two reviews during the year (66), placing special emphasis upon the cellular distribution of the vitamin as revealed by the acid silver nitrate staining technique. Bournes' extensive work with essentially the same approach has provided similar data. There remains, however, some question concerning the degree of interference by minute amounts of other constituents of the cell. If the silver deposition is a valid measure of ascorbic acid in all the cells studied, then one must conclude that such organisms as bacteria and yeast normally contain the vitamin. In typical cells, it appears to be well established that ascorbic acid is closely associated with the Golgi apparatus and mitochondria.

A number of papers have added further evidence in support of the viewpoint that vitamin C is of major importance in the detoxifi-

cation processes. Earlier papers from the author's laboratory and from other laboratories have shown that injections of toxins or other toxic materials may cause a marked depletion of vitamin C from the tissue, and that animals whose tissues are moderately or severely depleted of their vitamin-C reserves are subject to greater injury by toxin injections. Macy and associates (67) observed that infants who were receiving a limited and constant supply of the vitamin showed no signs of scurvy normally, but when they were subjected to the added stress of minor infections, indications of clinical scurvy appeared. Later the signs of scurvy disappeared spontaneously when the effect of the infection had passed. Kaiser & Slavin (68) observed a significant correlation between low blood ascorbic acid values and severe infections with hemolytic streptococci. An impressive group of papers reported that active tuberculosis created a demand for extra quantities of vitamin C (69). Warns (69), for example, found that the daily requirement to establish normal excretion was increased three to five times in patients with tuberculosis. Increased requirements were also observed in large groups accompanying rheumatic fever (70), peptic ulcer (71), and artificial fever induced by heating in a chamber (72). Four papers (73) were in agreement that hyperthyroid activity created a greater demand for vitamin C. The reported functional relation between adrenalin and ascorbic acid was shown by Daoud and associates (74) to be due to the nonspecific acidic effect of large doses of the vitamin when given by injection. Like many of the earlier and erroneous reports of direct *in vitro* detoxification, the reported effect was not observed when adequate care was exercised in relation to pH change. The results of Leibowitz & Guggenheim (75) are in agreement with the reviewer's experience in relation to *in vivo* detoxicating effects of ascorbic acid, but they are not in agreement (Sigal & King, 1937) on the point that "in all cases (diphtheria toxin, virus, phenol) the effect was shown by a real chemical combination between vitamin C and the poisons."

Vedder & Rosenberg (76) observed that when rats were given large doses of jewfish oil (100,000 I.U. of vitamin A), toxic symptoms resembling scurvy were induced, and vitamin-C excretion dropped to very low values. Administration of 5 mg. of ascorbic acid per day protected the animals almost fully. Protective action by the vitamin against phenylquinolinecarboxylic acid poisoning was demonstrated by Borsetti (77). The minimum lethal dose in guinea pigs was raised 25 to 65 per cent by the injection of 50 mg. of ascorbic

acid per day, and the toxic material was observed to deplete the vitamin from the tissues. Excessive amounts of copper were found by De Caro (78) to induce a higher demand for vitamin C in guinea pigs.

Ecker and associates (79) continued their studies of the close relation between vitamin-C deficiency and inactivation of guinea pig-blood complement (a reversible phenomenon). Chu & Chow (80) observed the same relation in a brief study of human blood. Madison *et al.* found a variable relationship between the vitamin-C intake of guinea pigs and their rate of developing precipitins (81), and Hasimoto *et al.* (82) reported that the anaphylactic reaction of horse serum in guinea pigs was greatly reduced by administering vitamin C.

Two papers during the year provided further evidence that vitamin P, reported by Szent-Györgyi, does not exist (83), at least in the sense of being an essential factor for guinea pigs.

*Human requirement and excretion.*—A number of good papers appeared during the year, relative to (a) clinical methods of measuring the state of nutrition, and (b) surveys of the incidence of malnutrition in typical population groups. The capillary fragility test as developed by Göthlin and associates (84) has continued to serve as a very useful means of detecting moderate to severe states of depletion, even though it is recognized that such factors as infections greatly alter the reliability of the test. As chemical methods for the direct measure of vitamin concentration in body tissues *in vivo* are studied in more detail, it becomes evident that each experimental method is subject to unaccountable variations from time to time. Hence, it is fortunate that a number of methods are becoming available.

There is general agreement that blood values below about 0.5 mg. per cent indicate a severe state of depletion, and that values ranging up to about 1.0 mg. per cent indicate mild deficiencies. For normal, well-nourished people, 1.2 to 1.5 mg. per cent is a common range of concentration. Minimum levels of intake to protect infants and adults from scurvy may be stated as approximately 10 to 25 mg. per day, respectively, but it is evident that the requirements for maintaining a "normal" state of nutrition are more nearly 25 and 50 mg. respectively, with advised standards of 50 and 100 mg. or more per day to provide for "optimum" nutrition and defense of the tissues against toxins, etc. The data from different laboratories for blood titration values were in reasonably good agreement (85). Barron

*et al.* (86) used a technique in which 10 mg. of ascorbate per kilogram of body weight was injected, providing a basis for measuring initial and subsequent blood values and urinary excretions (three-, six-, and twenty-four-hour periods). They placed greatest value on the per cent excretion within twenty-four hours after injection. The values reported for ascorbic acid in spinal fluid (87) were in general agreement with the values given for blood.

Urinary excretion after feeding 100 to 500 mg. per day provides a good indication of the state of nutrition in most individuals, but it is evident that there are great variations in the intake-excretion ratio when large numbers of individuals are studied under comparable conditions. Excretion varies widely also when different individuals are given a constant dietary (88). Mawson (89) recorded an interesting case in which the kidney threshold was apparently extremely low. The intradermal test proposed by Rotter, based upon decolorizing an injected solution of indophenol in five, ten, or more seconds (normal, subnormal, or deficient, respectively) has met with varying acceptance (90). By tissue analysis from autopsy cases, Giroud *et al.* (91) found further evidence that moderate to severe deficiencies are fairly common, even where there is no record or external indication of scurvy. The human requirement for vitamin C was reviewed by Smith (92), Dalldorf reviewed the pathology of vitamin-C deficiency (93), and Abt & Farmer reviewed the pharmacology and therapeutics of the vitamin (94).

*Chemical reactions in animal tissues.*—Although the chemical behavior of vitamin C *in vitro* is characterized by its rapid rate of oxidation, the vitamin is relatively stable in animal tissues. From the histologic work of Bourne and of Giroud, and from other evidence, there is reason to associate the vitamin with lipid metabolism. The work of Wolbach and associates also indicates a close relation between the vitamin and special phases of protein metabolism. Many investigators have emphasized the probable rôle of the vitamin as a hydrogen carrier in tissue respiration, but a number of arguments can be cited against the rôle of the vitamin as a major respiratory carrier. Probably the strongest single argument against its carrier rôle is the fact that the tissues increase rather than decrease in respiratory rate when the vitamin concentration is decreased. Addition of the vitamin to the deficient tissue *in vitro* does not significantly affect the respiratory rate.

Stotz *et al.* (95) found that guinea pig-liver brei catalyzed the

oxidation of ascorbic acid very slowly. The agent responsible for the catalysis was apparently cytochrome (and cytochrome oxidase). Glutathione exerted very little effect upon the oxidation rate, and in turn, ascorbic acid did not affect the rate of oxidation of glutathione in contact with the brei. Schultze *et al.* (96), in parallel with the oxidation studies, found that protein-sulphydryl groups in suspensions of liver, muscle, intestinal wall, whole blood, and erythrocytes rapidly reduced dehydroascorbic acid to ascorbic acid. Coagulation did not reduce the reduction rate, but it was blocked by iodoacetate, alloxan and arsenite—agents that block the reactivity of sulphydryl groups. Glutathione in unwashed suspensions also served to reduce the reversibly oxidized acid. In the presence of an excess of ascorbic acid, however, as in normal animal tissues (high ascorbic: dehydroascorbic ratio), very little reduction by glutathione or protein-sulphydryl groups occurred. There was no evidence of a carrier function through the dehydrogenases in animal tissues. In summation, the authors concluded that “there is considerable evidence against, and little evidence for, the function of ascorbic acid as a major respiratory agent in animal tissues.” From consideration of his own unpublished work and published data, Barron stated (86) “we postulate that ascorbic acid does not form part of a series of catalysts acting in cellular respiration.”

Lemberg *et al.* (97) published a series of papers on the rôle of ascorbic acid, through coupled oxidation reactions, in converting protohematin to verdohematin. The relation of ascorbic acid to phosphatases has been studied, but with widely differing conclusions in different laboratories (98). A possible relation between ascorbic acid and esterase activity was indicated by the work of Raabe and associates (99). Tauber (100) published a review of the relation of ascorbic acid to various enzyme systems.

A marked effect of lipid feeding upon vitamin-C excretion by the rat was observed by Musulin *et al.* (101). In contrast to Mentzer's results (102), the authors found that rats placed on inanition after receiving a diet of Purina chow or a diet of oats, bran and milk powder showed a sharp drop in ascorbic acid excretion within three to four days. When placed on a milk diet, growth was resumed but the excretion of vitamin C remained low, about 0.3 mg. per day. Oats, or the unsaponifiable matter from oat oil caused a rise in excretion from 0.3 to 2.0 or more mg. per day. The amounts of vitamin C in the urines were verified by biological assay, based in turn



upon titration values. The volatile fraction from halibut-liver oil produced a similar effect, but feeding the common sugars, proteins, or a number of vegetable oils did not cause a significant increase in the rate of excretion. The authors refrained from stating whether the observed effect was primarily dependent upon conversion of the lipid to ascorbic acid, or to a stimulating effect upon synthesis of the vitamin. The technique offers a valuable new lead in relation to the rôle of vitamin in animal physiology however.

A number of papers that are of interest, but omitted from specific comment because of lack of space, are listed together with titles (103) at the end of the bibliography.

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## FAT-SOLUBLE VITAMINS\*

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### VITAMIN A

*Studies relating to vitamin A<sub>2</sub>.*—In the last review<sup>1</sup> it was noted that probably there were two forms of vitamin A, now generally referred to as "vitamin A<sub>1</sub>" and "vitamin A<sub>2</sub>." The compound formed by treatment of vitamin A<sub>1</sub> with antimony trichloride has an absorption maximum at 620 mμ while the antimony trichloride compound of vitamin A<sub>2</sub> obtained from fresh-water fishes has an absorption maximum at 693 mμ. Rather extensive investigations of the distribution of these two forms of vitamin A have led definitely to the conclusion that vitamin A<sub>1</sub> predominates in the tissues of salt-water fishes and vitamin A<sub>2</sub> predominates in the tissue of fresh-water fishes.

Gillam, Heilbron, Jones & Lederer (1) studied the ratio of the substances that have absorption maxima at 693 and 620 mμ and found the ratios to be 2:1 and 0.15:1 respectively in the liver oils from fresh-water and marine fish from Russian waters. These ratios show a considerable range of variation in different tissues, such as eyes, intestines, and pyloric caeca of various species of fish. Edisbury, Morton, Simpkins & Lovern (2) found a high concentration of vitamin A in oils prepared from tissues of the alimentary tract especially from the pyloric caeca which they reported for the cod to be ten to twenty times greater than that of average cod-liver oil. These observations concerning the presence of vitamin A in the intestinal wall have led to speculations concerning a possible rôle of the vitamin in assimilation of fat from the intestinal tract, but there are as yet no definite data supporting these hypotheses. Gillam and his associates (1) came to the conclusion that concentrates of the chromogens having absorption maxima at 693 and 620 mμ have approximately the same biological activity.

Investigations have shown that the livers of mammals, birds, and reptiles do not contain vitamin A<sub>2</sub>. However, it has been demon-

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<sup>1</sup> *Ann. Rev. Biochemistry*, 7, 338 (1938).



There are suggestions in the literature of a third chromogen (1, 5) having an absorption maximum at 285 m $\mu$ , and Lederer & Rathmann (7) have observed a second band at 645–650 m $\mu$ . This suggested to them that the ratio of the absorption of the two bands—E at 693/E at 620 = 2—in the oil from livers of fresh-water fishes does not correctly represent the ratio of A<sub>2</sub> to A<sub>1</sub> in that the band at 645–650 m $\mu$  overlaps the band at 620 m $\mu$ . Further investigations are necessary to substantiate this conclusion.

*Dark adaptation and vitamin-A requirement.*—Attempts to determine vitamin-A requirements in man by making use of improper dark adaptation of the eye have led to further studies of the instruments and methods used in detecting the physiological response of the eye to different intensities of light and correlating this with vitamin-A deficiency.

The reason that dark adaptation can be used as a measure for vitamin-A deficiency lies in the chemical relationship existing between vitamin A and visual purple in the eye. The rate of regeneration of visual purple which is bleached by bright light is dependent upon the adequacy of vitamin A in the organism. When there is a deficiency of vitamin A, regeneration takes place much more slowly. The instruments used are designed primarily to measure the rate of regeneration of visual purple.

The so-called biophotometer has been used extensively in studying dark adaptation. Palmer & Blumberg (8) have pointed out some definite limitations in the use of this instrument, particularly as applied to determining the vitamin-A requirement of children. They believe there is a learning factor which influences the results obtained and they do not believe that biophotometric tests are adequate for determining the presence of mild degrees of vitamin-A deficiency in school children. Schuck & Miller (9) reported biophotometric tests on ninety-four college women and stated that the variability in their results suggested that further work should be done to determine the response of individuals known to have a satisfactory vitamin-A intake. The investigations of Isaacs, Jung & Ivy (10) throw further doubt on the reliability of the biophotometer in detecting vitamin-A deficiency. They studied medical students whose vitamin-A intake by calculation ranged from 1,650 to 9,725 International units per day. Definite correlation between biophotometric readings, dietary vitamin A, and clinical symptoms of vitamin-A deficiency could not be obtained. Snelling (11) was unable to observe any consistent improve-

ment in dark adaptation in a group of thirty-four school children after receiving large supplies of vitamin A in the form of percomorph oil even though seventeen members of the group were abnormal, and fourteen considered borderline cases before vitamin-A administration was begun. The necessity for careful and frequent calibration of the biophotometer is stressed in a paper by Booher & Williams (12). These investigators fed daily doses of 5,000 to 8,500 International units of vitamin A to thirteen of 106 employees of the United States Department of Agriculture who showed the poorest dark adaptation. Five of these individuals responded to the increased vitamin-A intake and eight (one of whom was known to be afflicted with congenital nightblindness) showed no improvement.

Corlette, Youmans, Frank & Corlette (13) found twenty of fifty ambulatory adults to have poor dark adaptation. When these subjects were treated with vitamin A in the form of halibut-liver oil, all but one showed improvement. Ahmand & Harris (14) observed two of thirty boys receiving 1.5 pints of milk daily and also fish-liver oil to have subnormal dark adaptation. With the administration of additional vitamin A these individuals showed normal response.

Hecht & Schlaer (15) introduced several refinements in the instrument for measuring dark adaptation which they call the "Adaptometer." They present evidence to show that the cones of the eye are affected by exposure to intense light as well as the rods. The recovery after exposure begins earlier and is more rapid in the cones. These authors (16) found that thirteen out of fourteen patients, suffering from alcoholic cirrhosis, showed disturbance of dark adaptation. Two of these were given 40,000 U.S.P. units of vitamin A daily and one became normal in nineteen days. The other needed a tenfold increase in vitamin intake for complete recovery. Four normal (17) individuals were observed on normal, low and high vitamin-A intakes extending over a period of several months. The most striking aspect of these studies was the almost parallel behavior of the rods and cones during recovery periods. The investigators feel that this parallelism indicates that vitamin A is probably associated with visual violet or iodopsin formation in the cone in much the same manner as it is associated with visual purple formation. The photosensitive pigment "iodopsin" has been isolated from the cones of the retina of chicken eyes (18). There is also evidence of the presence of a photosensitive substance in the eye of the frog (19).

In studying the relation of dark adaptation to vitamin-A deficiency

it must be recognized that as yet no normal threshold of dark adaptation has been established and that wide variations among individuals exist. Light-eyed individuals (20) are more sensitive to bright light and have poorer vision in low illumination than dark-eyed persons. Prolonged or slow dark adaptation is also associated with a number of diseases: congenital nightblindness has long been recognized.

Several investigators (8, 9, 10, 11, 12, 33) have discussed the inconsistencies of the results obtained and the reliability of criteria used in determining vitamin-A deficiency by dark-adaptation methods. These discussions show that the methods are still in the developmental stage and indicate that further studies in this field are necessary in order to determine their reliability.

Friderichsen & Edmunds (21) have studied the minimum amount of light to which infants will respond as a criterion of vitamin-A balance. They found that when infants were receiving daily a certain quantity of vitamin A, an increase in the amount had no effect on the irritability of the eye; but if the amounts were decreased the eye became more sensitive to the same intensity of light. The investigators believe that this procedure lends itself to determining vitamin-A deficiency in infants from one to twenty-four months of age.

An infrared photographic method (22, 23) has been used in studying the rate of pupil dilation of the rabbit during dark adaptation. Perhaps this procedure may afford a means of detecting changes due to vitamin-A deficiency.

Through dark-adaptation studies on prisoners in Denmark, Edmund & Clemmesen (24) came to the conclusion that adults receiving daily slightly less than 1,400 International units of vitamin A over a period of nine months show no evidence of vitamin-A deficiency.

*Utilization of precursors of vitamin A.*—Previous investigations have shown that a difference exists in the capacity of animals of different species and even different breeds of the same species to convert carotene into vitamin A. The extent to which precursors can be utilized has received further study.

Lanzing & Van Veen (25) and Spruyt & Donath (26) studied the distribution of carotenoids in various fruits and vegetables generally used in the native diets of the Dutch East Indies. As is to be expected they found considerable variations in the same fruit depending upon stage of maturity, variety and other factors. The same investigators (27) then studied the distribution of these carotenoids in the blood of seventy-four prisoners who were receiving 9,000 Inter-



national units of vitamin A in their vegetable diet. Blood analyses revealed the presence of 7.9 International units of vitamin A per 10 cc. of serum and also 8.6  $\mu$ g. of total carotenoids. These values were practically identical with those found for Europeans in Batavia, and native servants (28) receiving a mixed diet containing abundant vitamin A derived from vegetables, animal fat and dairy products. While alpha- and beta-carotene occurred in the highest concentration in the food they were found in low concentration in the blood indicating almost complete conversion of these carotenoids into vitamin A. On the other hand cryptoxanthin, which formed only 10 per cent of the total precursors of vitamin A in the diet, was found in larger quantities in the blood. The authors believe these results indicate that human beings may have difficulty in utilizing cryptoxanthin as a source of vitamin A.

That carotene in spinach and carrots may be poorly utilized is indicated in a report by Van Eekelen & Pannevis (29). It was observed that 94 to 99 per cent of the total carotene ingested was excreted in the feces while only 41 per cent of the carotene dissolved in oil was excreted. Whether this difference is due to absorption or destruction of the carotene is not apparent. Studies on dogs (30) indicate that carotene from carrots is utilized as readily as carotene in oil, or vitamin A in cod-liver oil, when the intake is 20 International units of vitamin A per 100 gm. of body weight.

The blood of eleven infants and sixteen young children suffering from xerophthalmia was studied by Haas & Meulemans (31) to determine vitamin-A and carotenoid content. Six of the eleven infants and thirteen of the sixteen children showed no vitamin A in the blood. The infants had a carotenoid content varying from 12 to 46  $\mu$ g. per 100 cc. of blood and in the children the range was from 8 to 29  $\mu$ g. The authors believe that vitamin-A content of the blood is a better criterion for judging vitamin-A deficiency than carotenoid content.

Clausen & McCoord (32) have made extensive studies of the vitamin-A, carotene and xanthophyll content of the blood of different age groups of infants up to two years to determine the normal variations and changes due to disease. The carotene content is lowest at birth and at that time is exceeded by xanthophyll. This ratio is reversed later in life. Vitamin A is also relatively low at birth. Carotene increases in the blood because it is rapidly absorbed from the diet but the rate of absorption is slower than for vitamin A. The vitamin-A, carotene and xanthophyll content of the blood is decreased during

infections due in part to low intake and in part to fever. When body temperature returns to normal after fever the vitamin-A content of the blood may temporarily go above normal.

Compounds other than carotene can be converted to vitamin A by the animal organism (34). Beta-apo-2-carotinal was found to possess a biological activity at daily doses of 5  $\mu$ g. and the polyene alcohol obtained from this compound had biological activity when fed in daily doses of 10  $\mu$ g. Von Euler, Günther, Malmberg & Karrer fed beta-apo-2-carotinal to rats. When they examined the livers of these animals they found that the unsaponifiable fraction of the liver had an absorption maximum in the ultraviolet which was approximately the same as for vitamin A.

*Hypervitaminosis A.*—Opinions differ as to the toxicity of vitamin A when fed in massive doses. Weslaw, Wronski, Wroblewski & Wroblewski (35, 36) observed injury to rats by oral, subcutaneous and percutaneous administration of "Vogan" and "Cresavit." Injections were more than twice as active in impairment of tissue as oral administration. Typical symptoms of hypervitaminosis A were cachexia, loss of weight, fragility of the bones, skin changes—such as loss of hair—and inflammation of the eyes accompanied by xerophthalmia. The exact composition of the products used in these studies is not known so it is not possible to explain why these results differed from those obtained by Vedder & Rosenberg (37) and by Ikegaki (38). Vedder & Rosenberg fed jewfish-liver oils containing approximately 600,000 International units of vitamin A and approximately 5,000 International units of vitamin D per gram to rats weighing 50 gm. at the beginning of the experiment. The dosage of vitamin A ranged from 25,000 to 100,000 units daily. Evidence of injury was not observed and the authors concluded that "If vitamin A is ever toxic it is in excess of 100,000 International units daily for 50-gram rats." Ikegaki came to a similar conclusion after two to three months of subcutaneous or intramuscular injection of high doses of vitamin A in the form of "Biostearin." His studies were made on both rats and guinea pigs. Vedder & Rosenberg studies provided data which show that there must be considerable destruction of vitamin A when fed in massive doses, and the authors believe that this accounts for failure to observe toxicity. Papke (39) found symptoms of overdosage of "Vogan" in young rats when fed in dosages of 0.5 to 2 cc. daily. He observed spontaneous fractures and anemia. He failed to find similar symptoms in rats over six months of age. It is important to note that

he does not believe that the influence of vitamin D has been eliminated in these studies on "Vogan."

#### VITAMIN D

It is impossible in a brief review to do justice to all of the fields of investigation on vitamin D. It seems advisable therefore to limit this paper to the more important studies on the chemistry of this vitamin.

A number of papers have dealt with the relative efficacy of different forms of vitamin D in the treatment of different species of animals. For the most part the results of earlier investigations have been confirmed.

It is now apparent that vitamin D can be produced artificially or obtained from fish liver oils which has a greater or lesser antirachitic effect on chicks than the vitamin D of cod liver oil if the products are fed at the same unitage as determined by biological assays with rats. Consequently, the results obtained from feeding rats cannot be used for judging the value of vitamin-D products for poultry.

On the contrary there appears to be no convincing evidence that man responds very differently from the rat so that this animal can be used to determine the potency of materials intended for human consumption.

There are reports which may lead to the conclusion that the vitamin D produced from 7-dehydrocholesterol is more efficacious in the treatment of rickets than the vitamin D produced from ergosterol. Since small differences in antirachitic effects are difficult to establish in clinical studies these results need further confirmation.

Evidence is accumulating that vitamin D plays a definite rôle in the prevention of dental caries.

*Chemistry.*—Hickman and his associates have extended their investigations of the chemical nature of vitamin D by means of molecular distillation of concentrates from fish-liver oils. Hickman & Gray (40) determined first the elimination curve for pure calciferol when dissolved in a cod-liver oil especially treated to remove interfering substances. They recovered 95 per cent of the added vitamin and the curve had a peak at 146° C. By a similar procedure they were unable to demonstrate a smooth distillation curve for vitamin D<sub>3</sub> but found a maximum in the curve at about 150° C. By assaying the different fractions from the distillation of vitamin D from cod-liver oil evidence was obtained for the existence of at least four and possibly six forms of vitamin D. Most of the vitamin D consisted of two

forms which gave maxima in their elimination curves close to the median temperature for distillation of vitamin D. The lowest boiling fraction of vitamin D when assayed by Bills, Massengale, Hickman & Gray (41) was found to be from one-half to one-fourth as effective for chickens as the total vitamin D of cod-liver oil when compared on the basis of rat-units. Liver oils obtained from other fish, such as spearfish and white sea bass, when subjected to analysis by distillation, yielded elimination curves distinctly different from each other and from the one obtained with cod-liver oil. By adding pilot dyes, whose distillation curves had previously been determined, to the distillation mixtures, the authors obtained evidence that a difference of  $\text{CH}_2$  in the side chain makes a difference of approximately  $4.5^\circ \text{C}$ . in the distillation temperature. This fact enabled them to estimate from the temperature of distillation the size of the vitamin-D molecule in various fractions.

Brockmann & Busse (42, 43) crystallized a vitamin from tunny-liver oil which they identified as the vitamin D produced from 7-dehydrocholesterol. The extinction curves, melting points, and analytical values of the two agreed. Brockmann & Busse disagree with Zucker and coworkers (44) that vitamin D from tunny-liver oil has a lower molecular extinction coefficient than vitamin  $\text{D}_3$  and a lower biological assay value than 40,000 International units of vitamin D per milligram. Brockmann & Busse believe that their previous assumption is correct, that the low molecular extinction values for vitamin D from tunny-liver oil were due to partial destruction of the ester during saponification.

In view of the practical importance of the synthesis of the different forms of vitamin D, it is noteworthy that Milas & Heggie (45) have reported (in a brief communication) the production of vitamin D from cholesterol. They treated the acetate with benzoquinone in a sealed tube for two hours at a temperature of  $120$  to  $130^\circ \text{C}$ . After some purification the crude product was exposed to ultraviolet radiation and upon biological assay was found to have a vitamin-D potency of more than 6,500 U.S.P. units of vitamin D per gram. Eckhardt (46) was unable to obtain 7-dehydrocholesterol from 7-amincholesterol or from some of its derivatives.

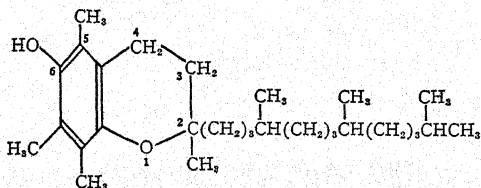
As a result of rather extensive chemical and physical studies on the structure of the cyclic portion of the molecule of vitamin D, Auwers (47) has concluded that the position of the double bonds in the formula proposed by Windaus is correct.

## VITAMIN E

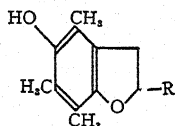
In 1922 Evans & Bishop discovered a dietary factor, subsequently called "vitamin E," which is particularly concerned with reproduction in the rat. A deficiency of vitamin E in the female rat does not interfere with oestrus, ovulation, or impregnation of the ovum, but the deficiency causes death and resorption of the fetus before maturity is reached. If a female rat on a vitamin-E deficient diet is given a single dose of vitamin E a few days after mating, or given sufficient vitamin E during the gestation period, normal young are produced. A deficiency of the vitamin in the male rat causes degeneration of the germinal epithelium which results in sterility. This damage appears to be irreparable.

Now that substances have been found which possess vitamin-E activity much attention has been directed to the elucidation of their chemical nature and structure. At least two naturally occurring substances, alpha- and beta-tocopherol, have been shown to have vitamin-E activity. Evidence has been presented to support the view that cumotocopherol and neotocopherol, described by German and Swiss investigators, are identical with beta-tocopherol (48). Prior to 1938 thermal decomposition indicated that the vitamin may be the monoether of either durohydroquinone or cumohydroquinone.

*Chemistry.*—Bergel, Todd & Work (49) through the synthesis of cetyl and allyl ethers of durohydroquinone and  $\psi$ -cumohydroquinone showed that alpha- and beta-tocopherols were not simple alkyl monoethers. They suggested that the properties of tocopherols could be best explained by assuming that in addition to the quinone ring there is a heterocyclic structure and stated that they were investigating the possibility of tocopherols being coumaran or chroman derivatives. John *et al.* (50) reached the same conclusion with respect to the structural relationship between tocopherols and coumaran or chroman. By studying the products formed by oxidizing alpha-tocopherol with chromic acid, Fernholz (51) concluded that this compound had a chroman structure and suggested the following structure which is based on the empirical formula,  $C_{28}H_{50}O_2$ .



He states: "α-Tocopherol is regarded as a substituted 6-hydroxy-chromane with a long aliphatic side chain attached to the pyran ring." Drummond and coworkers (52) found evidence for the existence of an oxygen ring in beta-tocopherol and suggested three possible formulas which were in accord with their experimental results. After further investigation, Moss & Drummond (53) arrived at the formula suggested by Fernholz. Bergel, Jacob, Todd & Work (54) synthesized two coumaran esters which were similar to beta-tocopherol in absorption spectrum, reducing properties, thermal decomposition products, and surface film measurements (117) and proposed the following structural formula for the coumaran portion of the molecule based on the empirical formula  $C_{28}H_{48}O_2$ .



They leave the configuration of the side chain to be determined.

Later synthesis by these investigators of coumaran and chroman derivatives verify the above conclusion (55, 56).

They further state that the formula for beta-tocopherol ( $C_{28}H_{48}O_2$ ) differs from alpha-tocopherol ( $C_{29}H_{50}O_2$ ) by one less  $CH_2$  group and that the compounds are simple homologues.

The synthesis of alpha-tocopherol through condensation of trimethyl hydroquinone and phytyl bromide in the presence of zinc chloride has been reported by Karrer, Fritzsche, Ringier & Salomon (57). The synthetic compound and the naturally-occurring alpha-tocopherol have the same absorption spectrum and reducing properties. The allophanates, 2,4-dinitrobenzoates and nitrophenyl urethanes of the substance when mixed with the same derivatives of alpha-tocopherol show no depression of melting points. The optically inactive synthetic compound can be resolved into two optically active forms by means of bromocamphorsulfonic acid. The sulfonate has the same optical activity as alpha-tocopherol. A single dose at 6 mg. was shown to have definite biological activity (58). These investigators state that according to the method of synthesis the product could have been either a chroman or coumaran but they believed the compound was a coumaran derivative (57). Later three different laboratories (54, 50, 59, 48, 60, 61, 62) on the basis of further syntheses and by analogy, came to the conclusion that Karrer's synthesis produced

substances with a chroman structure, which supports the view of Fernholz that alpha-tocopherol is a substituted chroman.

John and coworkers (50) prepared synthetic chroman and coumaran derivatives which showed properties similar to alpha- and beta-tocopherol, respectively. The 2,3,5,7,8-pentamethyl-6-hydroxychroman (M.P. 108° C.) had an ultraviolet absorption spectrum similar to alpha-tocopherol. The 2,4,6,7-tetramethyl-5-hydroxycoumaran (M.P. 132° C.)—see Bergol, Jacob, Todd & Work (62)—had previously been found to be similar to beta-tocopherol.

It may be stated that alpha-tocopherol ( $C_{29}H_{50}O_2$ ) appears to have a chroman nucleus and beta-tocopherol ( $C_{28}H_{48}O_2$ ) a coumaran nucleus, and the exact nature of the side chain for these compounds has not been determined, but the structure suggested by Fernholz seems to be accepted provisionally.

In attempting to synthesize the tocopherols, a large number of compounds have been prepared which have the biological properties of vitamin E (63, 64, 65). Some of these compounds are much simpler in chemical constitution than the naturally-occurring tocopherols. On the contrary, Karrer & Jensen (67) have demonstrated that 2,5,7,8-tetramethyl-2-[4'-8'-dimethyl-nonyl]-6-hydroxychroman which is very similar in constitution to alpha-tocopherol is wholly inactive when fed in 20 mg. doses. Since the difference between the synthetic compound and alpha-tocopherol is only in the side chain, they conclude that the nature of the side chain influences the vitamin-E activity. They also refer to their previous investigations showing that the presence of methyl groups on the benzene ring have an important bearing on the biological potency of synthetic compounds. John, Günther & Schmeil (68) in experiments on the synthesis of chroman derivatives with the ring system of alpha-tocopherol showed that the ring did not possess biological activity but that the side chain appeared to effect the biological activity. No synthetic compound has been observed to have greater biological activity than either the synthetic or the naturally-occurring tocopherols. The other compounds have been administered only in comparatively large quantities.

Isler (69) reported that the acetyl derivative of *dl*-alpha-tocopherol is more stable to oxidation than the original compound.

Investigations dealing with the isolation of vitamin E from wheat-germ oil have been reported by Moss & Drummond (53), Karrer & Salomon (71), and Mackenzie, Mackenzie & McCollum (72).

*Biological assays.*—A critical review of biological assays for vita-

min E was published by Palmer (73) and this subject has been discussed further by Bacharach and his associates (74, 75, 76, 77, 78, 79, 80). Bacharach has suggested an improvement in the method of assay by using virgin rats. He has observed that rats which have gone through a gestation-resorption show a lower fertility than virgin females and also that the requirement of virgin females for vitamin E may be only one-fourth or one-fifth as great as that of females which have had a resorption of a litter due to vitamin-E deficiency. He is of the opinion that resorption may produce toxic products which cause damage to the female that is not entirely reparable. Bacharach advocates the use of a standard in vitamin-E assays, which is a cardinal principle in biological assays, and he also stresses the desirability of plotting results in the form of response curves to provide for more accurate analysis of data. He reports that 1.2 mg. of alpha-tocopherol and 1.9 mg. of beta-tocopherol have approximately the same vitamin-E activity. Mason & Bryan (81), as the result of a rather extensive investigation, have concluded that animals can be obtained which more uniformly show vitamin-E deficiency by putting mother and young on a vitamin-E ration at about the middle of the lactation period.

A colorimetric method (82) for the determination of tocopherol, which depends on the ability of the compound to reduce ferric chloride, has been proposed by Emmerie & Engel. Their results were in good agreement with the determination of alpha-tocopherol by potentiometric titration with gold chloride as proposed by Karrer (83).

*Requirement of different species.*—Thomas and associates (84, 85, 86) have made an important contribution on the vitamin-E requirements of different species of animals. They destroyed the vitamin E in the rations by treatment with ferric chloride and demonstrated that these rations did not contain sufficient vitamin E to promote reproduction in rats. Goats kept on such rations reproduced normally and the investigators succeeded in producing three filial generations of animals on these rations. The observations led definitely to the conclusion that goats will reproduce normally when fed rations which are deficient in vitamin E. Goats which had been raised on the vitamin-E deficient rations were found to produce adipose and muscle tissue and milk which did not contain demonstrable traces of vitamin E, but the same tissues and the milk from goats raised under ordinary farm conditions were found to contain the vitamin. The authors consider their observations to give definite indications that the goat cannot synthesize



vitamin E. In preliminary studies they found that vitamin-E deficient rations do not interfere with reproduction in sheep or rabbits.

#### VITAMIN K

*General.*—The term "vitamin K" was proposed by Dam (87) as an abbreviation of the name "*Koagulations-Vitamin*" to apply to the substance that was necessary for the prevention of a nutritional-deficiency disease in chicks. Manifestations of the disease are: a delayed clotting time of the blood; and a hemorrhagic condition which could not be cured by the known vitamins, including vitamin C. Schönheyder (88) afterward demonstrated that the delayed clotting time was associated with a low prothrombin content of the blood which could be normalized by feeding preparations containing vitamin K. Other constituents of the blood known to be necessary for clotting were found to be normal. Investigations dealing with the properties of vitamin K (89) and attempts to isolate it have been reviewed previously (90, 91, 92). In the investigations referred to, the vitamin was found in the fat-soluble, unsaponifiable, nonsterol fraction of an ether extract of alfalfa. Lichman & Chambers (93) have recently reported, however, that they obtained from liver a sterol which reduced the clotting time of the blood of jaundiced dogs and rats, and the blood of chicks deficient in vitamin K. The relation of this substance to vitamin K needs further investigation before it can be properly classified.

The chick was used in the early experiments on the discovery, chemical nature, and attempts at isolation of vitamin K, and the pathology of vitamin-K deficiency. The duck, goose and pigeon (94, 95) have also now been shown to be subject to vitamin-K deficiency.

Although the pathological picture of slow blood clotting and hemorrhagic condition can be reproduced by biliary fistulas in the dog and rat, the attempts to produce vitamin-K deficiency in mammals have met with a certain degree of failure. This failure to produce vitamin-K deficiency in mammals (98, 99, 100) may be due to the possible synthesis of vitamin K in the intestinal tract. While this has not been demonstrated it seems to be entirely possible since vitamin K can be produced by bacterial action on such substances as fish meal and casein (96, 97).

*Clinical and experimental observations.*—These observations on vitamin K have led to a very important clinical application in the treatment of patients having hemorrhagic tendencies associated with

a low prothrombin content of the blood. The danger of post-operative hemorrhage in cases of obstructive jaundice has long been recognized. Quick (101, 102) observed that hemorrhage in such cases was frequently associated with a low prothrombin content of the blood and that in severe cases it may be as low as 10 per cent of the normal (103). As long as the prothrombin content of the blood remains about 20 to 30 per cent of the normal, there appears to be no great danger from hemorrhage, but when it falls below these levels the clotting time of the blood may be greatly delayed. The feeding of vitamin-K preparations, together with bile or bile salts, has been found to be effective in raising the prothrombin content of the blood above the danger level.

Brinkhous, Smith & Warner (104) reported that cases of obstructive jaundice and biliary fistula showing a hemorrhagic tendency associated with low prothrombin responded more rapidly to treatment with a combination of bile and vitamin-K preparations than with bile feeding alone. Very shortly thereafter a series of papers from the Mayo clinic reported similar observations (98). Dam & Glavind (100) observed that parenteral administration of vitamin K to jaundice patients is ineffective without concurrent feeding of bile salts. This suggests that obstruction of the bile interferes with the absorption of vitamin K from the intestine. In a later report by Snell, Butt & Osterberg (105) it was stated that the feeding of a vitamin-K preparation, together with bile salts, to thirty patients with obstructive jaundice, brought about normal coagulation time within twenty-four to seventy-two hours.

A tendency to bleed has been observed in dogs (99) with biliary fistulas of long duration. Such dogs are low in prothrombin. The prothrombin content may be raised by the feeding of bile alone, but it responds more rapidly to treatment with bile or bile salts, together with vitamin-K concentrates. Greaves & Schmidt (106) found the blood of rats with bile fistulas low in prothrombin and slow to clot. The feeding of vitamin-K preparations in massive doses relieved these symptoms. The feeding of bile or bile salts also decreased the blood-clotting time. Rats made icteric by ligation of the bile duct and fed a stock diet (free of bile salts) had a low prothrombin content of the blood. When bile salts were fed the prothrombin returned to a normal level. These observations supply proof that the bile salts aid in carrying vitamin K through the intestinal wall.

The literature (98, 107, 105) contains suggestions that vitamin-K

intake may be related to prothrombin deficiency in such conditions as sweet clover disease, liver injury from chloroform and other agents, but further study is necessary to demonstrate such a relation. The fact that the prothrombin content of the blood is low in these cases of liver injury suggests that somehow the liver takes part in the formation of prothrombin.

*Assay of vitamin K.*—At the present time the methods proposed for the assay of vitamin K involve the use of young growing chicks. Almquist (108) has described a preventive method in which the quantity of substance necessary to prevent the development of abnormal clotting time in day-old chicks is determined. Dam (109) proposed to determine the quantity of material necessary to restore the normal clotting time of the blood of chicks depleted of vitamin K. Later he (110) recommended as a standard the use of a preparation of dried spinach, to which a value of 500 units of vitamin K per gram was ascribed. The activity of the unknown material in curative tests was then compared with this standard, using a prescribed procedure for determining clotting time (111). A modification of the methods of Almquist and Dam have been proposed by Dann (112) and Thayer *et al.* (113). Ansbacher (114) has published a note which suggests that it may be possible to make curative assays in a period of a few hours.

*Occurrence of vitamin K.*—Vitamin K occurs rather generally distributed in nature. The following materials have been found to contain the vitamin: pig, dog and beef liver, fish meal, alfalfa, kale, carrot tops, tomato, hemp seed, soybean oil, egg yolk, rice bran, chick feces, dried human feces, dried alcoholic human feces, oat shoots and several bacteria (98, 115, 116).

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FOOD AND DRUG ADMINISTRATION  
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## METABOLISM OF BRAIN AND NERVE

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### OXIDATIONS IN THE NERVOUS SYSTEM

*Oxygen uptake of brain "in vivo" and "in vitro."*—Gerard (1) and Holmes (2) point out that the oxygen uptake of brain *in vivo* is much greater than that of brain examined *in vitro*, whether in the form of slices or as a mince. The indication would be that our present technique of examining brain tissue is inadequate to give results which reflect accurately the magnitude of chemical events taking place in brain *in vivo*. On the other hand it has to be borne in mind that the methods for the estimation of oxygen uptake *in vivo* are themselves only approximate, in view of the experimental difficulties in ascertaining the amount of blood flowing through the brain, and they may have yielded too high values. It is obvious that accurate estimates of the oxygen uptake by brain, and by various parts of the nervous system *in vivo* are urgently required. According to Lennox (3) the oxygen content of blood leaving the human brain is about 60 per cent of the saturation value and is less than that of blood leaving the face and the extremities. There seems to be a loss of approximately 8 cc. oxygen per 100 cc. of blood in its passage through the human brain. Taking an estimate of the flow of blood through the human brain to be about one litre per minute (4) and the weight of the human brain to be about 1400 gm., the  $Q_{O_2}$  is approximately 17.1, *i.e.*,  $[80,000 \times 60] / [280 \times 1000]$ . This value may be compared with an older figure for the oxygen consumption of dog brain cited by Himwich & Nahum (5) from which a  $Q_{O_2}$  of 39 has been calculated. The respiration of brain-cortex slices in a glucose medium falls off with time, much more so in the case of the rat than in the case of the guinea pig, and the rate of fall depends on the ionic concentration of the medium. With rat cortex a  $Q_{O_2}$  of 19.1 was found during the first 24 hours of an experiment [Jowett & Quastel (6)] and a higher figure would have been obtained had it been possible to measure accurately the oxygen consumption during the first few minutes of the examination of the tissue. There is little doubt that the optimal conditions for the examination of brain *in vitro* have not yet been achieved, but it would be well to postpone judgment as to the



value of our present *in vitro* methods until more confidence can be placed in the accuracy of the results obtained by the methods adopted at present with the living animal.

The respiratory quotient of brain *in vivo* is known to be about unity, which points to the probability that carbohydrate is the main fuel of the brain *in vivo*. This conclusion is supported by the fact that the oxygen consumption is approximately equivalent to the glucose disappearance in the passage of blood through the brain—assuming complete oxidation of glucose. Himwich & Fazekas (7) examined cerebral blood samples from the superior longitudinal sinus and arterial samples of the femoral artery of a dog anaesthetised with amytal. They found the glucose consumption by the brain to be 13 mg. per 100 cc. blood. This corresponds to an oxygen consumption of 9.7 cc. per 100 cc. blood, whereas the value found for the average difference between the oxygen contents of arterial and cerebral venous bloods was 9.3 cc. per 100 cc. blood.

*Glucose oxidation.*—It has been shown independently by Baker, Fazekas & Himwich (8) and by Jowett & Quastel (9) that glucose oxidation by brain does not necessarily involve the oxidation of lactic acid. This was accomplished by the use of differential inhibitors. The former workers used nicotine and nicotinic acid whose mechanisms of action on enzymic reactions are unknown; the latter used hydroxymalonate which is known to be an inhibitor of lactic dehydrogenase. Both inhibitors diminished the oxygen consumption of brain in presence of lactate to a greater extent than that in presence of glucose. Moreover, Shorr, Barker & Malam (10) have observed carbohydrate oxidation by brain in presence of sufficient iodoacetate to inhibit glycolysis and Baker (11) has made a similar observation using glyceraldehyde as inhibitor.

There is still little information as to the nature of the intermediate steps in glucose oxidation by brain. The oxidations of glucose and of lactic acid cannot be independent, for the addition of glucose to brain tissue already burning lactic acid does not increase the rate of oxygen uptake (12, 13) and presumably there is an intermediate common to the oxidations of both substances. Probably this is pyruvic acid. According to Elliott, Greig & Benoy (13) the addition of pyruvate diminishes slightly the respiration of rat-brain cortex in presence of glucose. The latter authors report that during the oxidation of glucose by brain, a trace of pyruvic acid is always to be found.

Although, as is well known, glucose is freely oxidised by minced

brain, the effect of freezing and grinding brain tissue is to destroy its ability to oxidise glucose [Mann, Tennenbaum & Quastel (14)]. Moreover, an extract of brain prepared by mincing, grinding and dispersing the tissue in nine volumes of distilled water is unable to oxidise glucose (15).

*Pyruvic acid oxidation and transformations.*—There is little difference between the rates of oxygen uptake by brain secured by pyruvate or by glucose (13, 16, 17). It may be seen from the results of Jowett & Quastel (17) and of McGowan (18) that the disappearance of pyruvic acid is greater than can be accounted for by the oxygen uptake assuming a complete oxidation of the pyruvate by the brain. This is due at least partly to lactic acid and partly to acetic acid formation. Elliott *et al.* (13) have found that in a mixture of glucose and pyruvate, in the presence of rat-brain cortex under aerobic conditions, most of the pyruvate disappearance can be accounted for by lactate formation—the pyruvate acting presumably only as a hydrogen acceptor. This is not the case in the absence of glucose. Krebs & Johnson (19) have shown that under anaerobic conditions two molecules of pyruvic acid undergo dismutation to acetic and lactic acids, a reaction which is also known to take place with bacteria. This has been confirmed by Lipmann (16) and Weil-Malherbe (26). Granting that this dismutation will take place under aerobic conditions, McGowan (18) has found that the lactic acid formed aerobically by minced pigeon brain in presence of pyruvate does not account for the extra disappearance of pyruvate not accounted for by the oxygen uptake. Long (20) has found that acetic acid is formed as well as lactic acid when minced pigeon brain respire in presence of pyruvate, but that the quantities produced would account only for 30 per cent of the pyruvate which disappears. The rest of the pyruvate is completely oxidised. The suggestion (19) has been made that the dismutation precedes the oxidation process so that the oxidation of pyruvic acid becomes in effect the oxidation of lactic acid produced by the dismutation. This is unlikely to be the case since Jowett & Quastel (9) have found that hydroxymalonate inhibits the oxidation of *D*-lactate and of *DL*-lactate by rat-brain cortex to a much greater extent than that of pyruvate (see also 26).

The respiratory quotient for brain respiration in presence of pyruvate is 1.22 for rat-cortex slices (13) and 1.30 for washed minced pigeon brain (18). The values are a little higher than that required for complete oxidation of pyruvate (see also 22).

Since it seems evident that pyruvic acid is not oxidised *via* lactic acid and that it is not oxidised (aërobically) directly to acetic acid, it is reasonable to turn to the possibility that pyruvic acid may undergo a preliminary condensation, oxidative or otherwise, before the main sequence of oxidative changes take place. The old theory of Toenissen and Brinkman that diketo adipic acid may be the first product of oxidation and that this breaks down *via* succinic acid has been much explored and has led to interesting results on succinic acid formation during brain metabolism (21, 22, 23, 26). Elliott & Greig (21) found very little succinic acid formation during pyruvate oxidation by chopped rat brain even in presence of malonate which is now well known to be an inhibitor of succinic acid oxidation. Weil-Malherbe (22, 26) found that succinic acid is formed when pyruvic acid or  $\alpha$ -ketoglutaric acid is added to brain under anaërobic conditions. Aërobically succinic acid is not formed by brain slices or minced brain, presumably owing to the activity of the succinic dehydrogenase present. When malonate is present succinic acid accumulates with minced brain tissue but not with brain slices. Whilst the fact that succinic acid formation can be demonstrated during brain respiration in presence of pyruvate is of undoubted importance and should throw some light on the mechanism of pyruvic acid transformation, the amounts produced in presence of malonate seem too small to make it very probable that succinic acid is in the main line of oxidative breakdown of pyruvic acid. Malic acid which is quickly formed aërobically from succinic acid by brain tissue is oxidised by this organ very poorly under aërobic conditions and a much higher rate of removal would have been anticipated to account for the complete oxidation of pyruvate and the lack of accumulation of malic acid. McGowan & Peters (24) do not agree with the view that succinic acid or  $\alpha$ -ketoglutaric acid is in the main line of oxidative breakdown of pyruvic acid. Acetopyruvic acid seems a possible important intermediate in pyruvic acid oxidation and Krebs & Johnson (25) have found that it is attacked by pigeon brain both aërobically and anaërobically. According to Simola & Alapeuso (105) brain tissue may transform pyruvic into citric acid, the amount being increased on addition of fumaric and malic acids.

It has been shown by Kritzmann (27) [see also Braunstein & Kritzmann (28)], that in brain tissue pyruvic acid reacts with glutamic acid to form alanine, a transference of the amino group taking place. This phenomenon serves to emphasise the highly reactive na-

ture of pyruvic acid in presence of brain. It seems quite probable that pyruvic acid may undergo other, as yet unknown, changes in brain which may throw light on the present very obscure line of oxidative breakdown of pyruvic acid. The possibility that acetaldehyde may be an intermediate has received no experimental support (29, 16).

*Lactic acid oxidation.*—The facts that aerobic glycolysis in brain is normally small and that glucose oxidation in brain can proceed independently of lactic acid formation, give a somewhat secondary importance to lactic acid as an intermediate in brain metabolism. However, this conclusion may have to be modified in view of Laser's finding (30) that diminished oxygen tension increases aerobic glycolysis in retina. Present methods which involve the use of tissue slices in 100 per cent oxygen may give values for lactic acid formation by brain *in vivo* which are too low. Himwich *et al.* (31) consider it doubtful whether lactic acid is utilised by brain *in vivo*. Their figures seem to make it unlikely that lactic acid is removed from the blood on its passage through the human brain. As is well known lactic acid is definitely oxidised by brain *in vitro*, pyruvic acid being demonstrable in small quantities (32, 17). The pyruvate concentration found with guinea-pig or rat-brain-cortex slices in a 0.02 *M* *D*-lactate medium is of the order  $2 \times 10^{-4}M$  (17). Quastel & Wheatley (33) have shown that lactate can be oxidised anaerobically by ferricyanide in brain slices, the oxidation being much accelerated by the addition of cozymase. A similar anaerobic oxidation of pyruvate does not take place, although methylene blue will bring about the anaerobic oxidation of pyruvic acid to acetic acid in presence of brain tissue (16; see also 96). Lactic acid is oxidised by an extract of rabbit brain in water (15) and it is concluded by Schoen & Gerard (34) that the oxidation of lactic acid is independent of the intermediate oxidation of succinic acid. They show that the oxidations of lactic and succinic acids in such an extract are independent. According to Zukhov (35), lactate, in the presence of oxygen, will restore the excitability of nerve poisoned by iodoacetate.

*Oxidation of various keto- and hydroxy-acids.*—Acetoacetate slightly increases the respiration of brain (24, 14, 19) and undergoes some reduction to  $\beta$ -hydroxybutyrate under anaerobic conditions (36).  $\alpha$ -Ketoglutarate is oxidised by brain slices (19) and by minced brain (24, see also 95, 96), but McGowan & Peters (24) point out that vitamin B<sub>1</sub> has no accelerating effect on its oxidation in presence of avitaminous pigeon brain. This keto acid, according to Krebs &

Johnson (19) and to Weil-Malherbe (22), gives rise to succinic acid when incubated anaerobically with minced pigeon brain; it is reduced in brain to *l*- $\alpha$ -hydroxyglutarate. The oxidation of the latter acid has been studied by Weil-Malherbe (37). Acetopyruvate is oxidised by pigeon brain (25) but  $\alpha$ -ketoadipate (24),  $\alpha$ -hydroxyacetoacetate (36), and  $\alpha$ -ketobutyrate (29) appear not to increase the respiration of brain. Phosphoglycerate (9) leads to only a slight increase in respiration of guinea-pig-cortex slices, indicating little or no transformation to pyruvic acid (compare muscle); it does not increase the respiration of brain in presence of  $\alpha$ -glycerophosphate. The dehydrogenase activating  $\alpha$ -glycerophosphate in brain has been studied by Euler, Adler & Günther (38) and Weil-Malherbe (39). *l*-Malate has a small effect in increasing the  $Q_{O_2}$  of brain slices (13); it is definitely oxidised in presence of brain slices anaerobically by ferricyanide so long as cozymase is added to the system (33). Oxalacetate is presumably formed. According to Elliott *et al.* (13) brain slices do not catalyse the decarboxylation of oxalacetate appreciably. Triosephosphate reduces cozymase in presence of brain (50).

*Oxidation of acetate and succinate.*—The addition of acetate has little or no effect on the respiration of brain (13, 14, 26). Succinate, which is well known to be easily oxidised by brain, undergoes rapid oxidation anaerobically by ferricyanide (33). The observation (40) that succinate is oxidised practically quantitatively to fumarate and *l*-malate is confirmed by Elliott *et al.* (13). That competition for oxidation takes place between succinate and other metabolites of the brain is suggested by recent experiments of Jowett & Quastel (17). Extracts of brain oxidise succinic acid rapidly according to Cohen & Gerard (15).

*Oxidation of glutamic acid.*—The dehydrogenase of this amino acid and its significance have been studied by Euler *et al.* (43).

*Oxidation of pentoses and inositol.*—*d*-Ribose and *l*-arabinose are not oxidised by brain slices of the rat [Dickens (44)]. Neither is the respiration of rat or rabbit brain increased by inositol [Young (41)].

*Oxidation of amines.*—The oxidation of the aliphatic amines, butylamine, amylamine, isoamylamine and heptylamine is accomplished by brain [Pugh & Quastel (46)]. The amine oxidase responsible for this oxidation oxidises also tyramine and indolethylamine (see also 51) and is presumably identical with tyramine and adrenaline oxidase (46, 47). Analysis of the products of amine oxidation shows that the corresponding aldehyde, ammonia and hydro-

gen peroxide are formed. The aldehyde may undergo a dismutation to the corresponding acid and alcohol (46). Indolethylamine oxidation in presence of brain as well as of the tissues is accompanied by the formation of a melanin-like pigment (46). The activity of amine oxidase is inhibited by a number of amines which are attacked by brain either feebly or not at all (46, 47), as, for example, ephedrine (49) or benzedrine (Pugh & Quastel, unpublished). Gaddum (48) discusses the possibility that some of the clinical effects of ephedrine administration (and presumably also of benzedrine administration) may be due to amine oxidase inhibition (see also 52).

*Oxidations by brain extracts.*—Extracts of brain prepared by grinding and dispersing the tissue in water are able to oxidise methylglyoxal,  $\alpha$ -glycerophosphate, lactate, fructose, succinate and *p*-phenylenediamine (15). According to Adler & Hughes (45) there exist triosephosphate and lactate oxidising systems in brain extracts but little or no glycerophosphate dehydrogenase.

*Cytochrome.*—The cytochrome absorption spectrum is given by cerebral cortex and central nuclei but not by white matter. No indophenoloxidase reaction is given by peripheral ganglia, but human substantia nigra gives a marked reaction and shows the *b* and *c* bands of cytochrome (252).

*Inhibitory action of narcotics.*—Jowett & Quastel (17) find that the respiration of rat and guinea-pig brain-cortex slices is inhibited by evipan, luminal and chloretone, in the presence of glucose, sodium lactate or sodium pyruvate, at concentrations of the same magnitude which produce narcosis *in vivo*. The inhibition of respiration by the narcotics tends to be independent of time when the potassium ion concentration of the medium is sufficiently high; at low potassium concentrations the inhibition increases with time. Both the oxidations of lactate to pyruvate and of pyruvate itself by brain slices are inhibited by narcotics. Ether exercises an inhibition of the respiration of brain-cortex slices in the presence of glucose, fructose, lactate, pyruvate and glutamate but none in the presence of succinate,  $\alpha$ -glycerophosphate and hexosediphosphate (6). The inhibition is greatest when glucose is the substrate. The inhibitory action of ether has a large temperature coefficient and quite clearly ether has much more pronounced irreversible effects than narcotics such as chloretone or the barbiturates. Brain respiration is affected by narcotics to a greater extent than that of other organs. Jowett (53) shows that ethyl urethane, magnesium, chloralhydrate, luminal, chloretone, evipan and

avertin all bring about measurable inhibition of brain-cortex respiration in presence of glucose at concentrations which induce narcosis. Alcohol behaves like ether in that brain respiration is progressively, and probably irreversibly, inhibited, and thus these substances behave differently from the other narcotics quoted whose inhibitions are known to be reversible. Hundhausen (54) could find no difference between the inhibitory effects of chloral hydrate or luminal on the respirations of brain cortex or brain stem. Binet & Strumza (55) conclude that chloralose has a direct action on the respiratory center. Haggard & Greenberg (56) point out that the toxicity of alcohol is influenced by the concentration of glucose in the blood. The higher the blood sugar the lower the toxicity of alcohol. According to Shapiro (57) intravenous administration of amytal may cause a marked lowering of the rate of oxygen consumption; according to Shaw, Steele & Lamb (58) ether anaesthesia causes a decrease in difference between the oxygen contents of arterial and venous bloods. Emerson *et al.* (59) point out that ether and divinyl ether anaesthesia may cause ketosis, indicating a disturbed carbohydrate metabolism (see also 60). Stone (61) shows that the brain lactic acid of anaesthetised mice is lower than normal (see however 64) and considers that this fact contradicts the view that during narcosis an inhibition of glucose, lactate or pyruvate oxidation may occur in the nervous system. It should be pointed out, however, that narcotics impair brain oxidation in quite a different way from cyanide which increases lactic acid formation in brain (61). The narcotics, unlike cyanide, do not interfere with the access to or activation of oxygen and appear to act to the greatest extent on the oxidation mechanisms involving glucose, lactate and pyruvate. The mechanisms by which the narcotics act on these systems are as yet unknown and the mechanisms by which lactic acid accumulates in the living brain are not yet clear. It is most probable that the amount of lactic acid in the brain at any time is dependent upon a variety of factors and until more knowledge has been secured as to the nature of these factors and how these may be influenced by extraneous factors, it is unsafe to conclude that a low value of lactic acid in the anaesthetized brain indicates that narcotics are not exercising inhibitive powers on the respiration of certain nervous centers. Mansfeld *et al.* (62) have carried out experiments on the respiration of slices of brain of anaesthetised animals and have found no diminution from the normal values. However, these experiments are open to the following criticisms: (a) any nar-

cotic in the brain slice would diffuse into the external medium and lower its effective concentration in the tissue; and (b) the technique adopted (62, 63) was such that the brain was not examined under optimal respiratory conditions, a lack of substrate being clearly present since the average value of the  $Q_{O_2}$  of brain was only 4.2. Narcotics in low concentrations have little or no effect on the respiration of brain in the absence of added substrates (17). Keeser (83), studying veronal distribution in brain, finds that it is chiefly confined to the thalamus and midbrain.

*Inhibition by amines.*—Tyramine, indolethylamine, benzedrine, and mescaline but not histamine inhibit the respiration of brain-cortex slices in the presence of glucose [Pugh & Quastel (46)]. Mescaline, which is oxidised by brain feebly or not at all (46), is oxidised by rabbit liver to the corresponding acid (65). Histamine has no inhibitory action on the respiration of brain-cortex slices (46). According to Loeper (66) amines, including tyramine, indolethylamine and histamine, may circulate in the blood under certain pathological conditions (e.g., liver disease and shock). Nieuwenhuyzen (245) has shown that intravenous injection of indolethylamine and other amines may give rise to a condition of catatonia. There is no choline oxidase in brain (67).

*Inhibition by cyanide and carbon monoxide.*—Carbon monoxide appears not to affect the respiration of retina but to increase markedly its aerobic glycolysis (68). Marshall & Rosenfeld (69) have studied the action of small doses of cyanide as a respiratory stimulant, showing that respiratory stimulation may occur in dogs and cats with no decrease in the oxygen consumption of the whole animal. They point out that stimulation from cyanide resembles closely that due to anoxaemia. It is reported that the respirations of brain (70) and of peripheral nerve (71) poisoned with cyanide may be increased by the addition of lactoflavin.

*Other inhibitors.*—Glyceraldehyde diminishes the respiration of brain in presence of glucose (14, 11) though it has little effect in the absence of glucose. According to Mendel *et al.* (72) the inhibition is due chiefly to *l*-glyceraldehyde. *p*-Aminonaphthol, at a concentration of  $10^{-4}M$ , inhibits brain respiration by 40 per cent (73). Sodium salts of higher fatty acids (e.g., decoate) have a similar effect (153). Lead (5 mg. lead acetate per 100 gm. tissue) reduces oxygen consumption of brain (74). Inhibitions by selenite are reported by Wright (77), and by maleic acid by Weil-Malherbe (151).



*Accelerators of brain respiration.*—Pyocyanine, according to Young (75), increases, at concentrations of  $10^{-3}M$  to  $10^{-5}M$ , the respiration of rat-brain cortex in presence of glucose. Oxidation in presence of lactate, fructose and pyruvate is also increased but when no substrate is added there is no increased respiration. Toxic fractions from *Bact. aertrycke* increase the oxygen uptake of rabbit-brain suspensions in the presence of glucose (76). Ammonium ions increase the respiration of guinea-pig cortex slices (151).

*Consequences of oxygen lack.*—McFarland, Knehr & Berens (78) have shown that under anoxia there is a decrease in efficiency of ocular movements; this is attributed to the delivery of a diminished amount of oxygen to the nervous tissue, subcortical as well as cortical. They believe that the changes in eye movements can be used to detect early effects of oxygen deprivation. Kraines (79) has shown that oxygen deficiency (breathing 10 per cent oxygen) leads to a diminution of intellectual function. Gellhorn finds that the administration of 3 per cent carbon dioxide offsets the decrease in visual discrimination due to oxygen lack and points out (80) that the carbon dioxide administration improves oxygenation of tissues in general and of brain in particular by a number of factors including an augmentation of the rise in blood pressure which occurs under conditions of oxygen want (81). The increased blood pressure due to oxygen want may be increased by insulin administration, the augmentation varying with the degree of hypoglycaemia (82). Inhalation of air containing 6 to 8 per cent oxygen raises the threshold of sciatic stimulation necessary to dilate the pupil in the rabbit (84).

*Effects of cardiazole treatment.*—Himwich *et al.* (85) have shown that during cardiazole convulsions an anoxaemia develops. The oxygen saturation of the blood falls to 42 per cent during various stages of the convulsions. It appears, therefore, that the effect of the convulsions is to reduce the oxygen supply to the nervous system (see also 86). Cardiazole itself has no effect on the respiration of brain tissue *in vitro* (87).

*Effects of insulin administration.*—Himwich *et al.* (225, 85) find that the oxygen utilisation of brain is considerably decreased (65 per cent) during hypoglycaemia induced by insulin and that the glucose utilisation is much diminished. Thus, hypoglycaemia brings about an anoxaemia in the nervous system although oxygen is freely available. Brain tissue of depancreatized or diabetic animals burns lactic acid and glucose at the normal rates (8) and addition of insulin to a

glucose-Ringer medium has no effect on glucose oxidation by brain (87). A series of insulin shocks tends to bring about degeneration of the nerve fibres of the encephalon (226).

*Vitamin B<sub>1</sub>*.—The literature for 1937 on the action of vitamin B<sub>1</sub> on brain metabolism has been reviewed by Peters & O'Brien (88). Ochoa & Peters (89) have studied the distribution of vitamin B<sub>1</sub> and cocarboxylase (vitamin-B<sub>1</sub>-pyrophosphate) in animal tissue including brain (see also 90) and have shown that there is less vitamin B<sub>1</sub> than cocarboxylase in the liver and brain. There is a larger relative increase in the cocarboxylase of brain than of other organs when avitaminous pigeons are dosed with vitamin B<sub>1</sub>. During avitaminosis the cocarboxylase of brain falls. Brain has only a very limited power of synthesis of cocarboxylase from vitamin B<sub>1</sub>. Ochoa (91) reports an activation of cocarboxylase action by vitamin B<sub>1</sub>. Galvão & Pereira (92) have made further studies on the oxidation of lactic acid in normal animals and in animals (fowls, rats) with avitaminosis B<sub>1</sub>. They conclude that the vitamin is indispensable for the oxidation of lactic acid in the central nervous system, but in avitaminosis B<sub>1</sub> the appearance of the chemical lesion may occur indifferently in any region of the central nervous system. The site of the lesion varies with the animal. Lipschitz *et al.* (93) conclude that inanition is not responsible for the inability of the brain to deal normally with pyruvic acid in polyneuritis. It is reported by Minz (94) that electric stimulation of the isolated pneumogastric nerve releases vitamin B<sub>1</sub>. Hirano (227) reports that the convulsions arising in polyneuritis disappear on injection of glutathione. Deficiency of dietary vitamin B<sub>1</sub> as a causative factor in the production of brain lesions by experimental alcoholic intoxication is described by Lhermitte *et al.* (244).

*Hyperthyroidism*.—Cohen & Gerard (97) state that the oxygen uptake of hyperthyroid brain is initially 30 per cent higher than normal and that it is increased approximately four times as much as that of normal brain on adding substrates involved in carbohydrate oxidation and glycolysis (glycogen, glucose, fructose, glycerophosphate, lactate, succinate). They conclude that "the absolute concentration of various enzyme systems is greater in hyperthyroid than in normal brain and that certain dehydrogenases are increased relatively more than oxidases."

#### CARBOHYDRATE METABOLISM

Information concerning work carried out during 1937 on glycogen breakdown in the brain and of the effects of hypoglycaemia

will be found in a review by Himwich (98). Kerr *et al.* (99, 42) have studied cerebral glycogen and its diminution during insulin hypoglycaemia. Cerebral glycogen is a precursor of lactic acid in the brain. Kerr (100) has isolated 36 mg. of glycogen from 100 gm. brain tissue, the carbohydrate being identical with liver glycogen. Glycogen in nervous tissue, both vertebrate and invertebrate, is reported by Havet (250).

The question as to whether glucose undergoes preliminary phosphorylation in intact brain before further metabolism takes place is still unsettled (see 137). Adler *et al.* (101) have shown that a cell-free preparation prepared by acetone precipitation of a brain extract is capable of causing glycolysis so long as adenylic acid, cozymase and a trace of hexose diphosphate are present. The glycolysis is inhibited by *dl*-glyceraldehyde (see also 104). The indication would be that a phosphorylation of glucose is necessary prior to glycolysis in a brain extract. Geiger (102) states that cytolysis of brain releases an inhibitor of glycolysis. An extract prepared at pH 5.5 to 6.0 contains a glycolytic system but no inhibitor. By the addition of a factor in boiled yeast or muscle extract, and of glutathione, creatine phosphate and cozymase a glycolytic activity is obtained which may be over twice that of the intact brain. Such a system also breaks down glycogen (but at a smaller rate than glucose) to lactic acid. Phosphate is esterified in a brain extract during glycolysis. According to Cori, Colowick & Cori (103) glucose-1-phosphate is formed from glycogen and inorganic phosphate in dialysed extracts of rabbit brain, the amount being increased by the addition of adenylic acid. Conversion of glucose-1-phosphate to hexose-6-phosphate proceeds more slowly than in a muscle extract. No synthesis of carbohydrate from lactate or pyruvate was observed by Benoy & Elliott (106) in presence of brain-cortex slices.

The effects of cations on glycolysis and respiration of brain are of interest, especially in view of the possibility that changes of permeability of the brain cell may play an important rôle in influencing the kinetics of brain metabolism. Quastel & Wheatley (107) have shown that calcium ions at low concentrations markedly increase anaërobic glycolysis and that strontium and magnesium have similar but smaller effects; potassium ions have a diminishing effect. These effects are the reverse of those found on brain respiration. It seems not improbable that the movements and effects of cations may become important for the interpretation of the Pasteur effect. A comprehen-

sive discussion of this phenomenon has been published by Burk (108). According to Kimura (109) the effects of cations on aerobic glycolysis depend on whether brain tissue is sliced or minced. Ammonium ions increase aerobic glycolysis of brain slices (151). Glutathione has no effect on the aerobic glycolysis of brain according to Baker (110) but Weil-Malherbe (151) finds that glutathione as well as glutamate, increases aerobic glycolysis of brain. Belitser (111) maintains that glycolysis may serve to restore or preserve the chemical components of the respiratory system. It is known from the work of Dickens & Greville (112) that brain slices kept anaerobically in the absence of glucose for a few minutes at 37° lose, or partially lose, their power to bring about anaerobic breakdown of glucose and it has been supposed that anaerobiosis in the absence of glucose brings about irreparable damage to the brain (2). Quastel & Wheatley (107) show, however, that if brain slices which have been kept anaerobically in the absence of glucose are subsequently exposed to oxygen their power of bringing about anaerobic glycolysis is regained.

Pertinent to the question of the mechanism of glycolysis is the work carried out on the anaerobic dismutation of pyruvic and  $\alpha$ -keto-glutaric acids (19, 26). The possibility that pyruvic acid is acting as a hydrogen acceptor in glycolysis seems to be supported by the observation that hydroxymalonate inhibits anaerobic glycolysis (9) both in the presence and absence of added pyruvate. The fact, however, that increasing concentrations of pyruvate do not, in the case of rat brain, diminish the effect of hydroxymalonate throws some doubt on pyruvic acid being the actual intermediary. The action of hydroxymalonate helps to explain the well-known inhibitory effect of lactate on anaerobic glycolysis (see, however, 151) *i.e.*, on the supposition that both substances compete with pyruvate for the lactic dehydrogenase in brain. Weil-Malherbe (151) has shown that anaerobic glycolysis of brain is inhibited by *d*- and *l*-glutamate, glutamine and *dl*- $\beta$ -hydroxyglutamic acid, the effect being reversed by the addition of pyruvic acid. He discusses the possibility that the effect may be due to a reaction between the keto acid and glutamate. Morgan & Friedmann (152) show an inhibition by maleic acid of anaerobic breakdown of glucose by brain (but see 151). Califano (113) finds that the glycolysis of retina is inhibited by agents which inhibit muscle glycolysis.

## NITROGEN METABOLISM

*Ammonia.*—According to Brühl (114) ammonia is a product of brain metabolism not normally to be detected in the cerebrospinal fluid. It may be found in the latter medium, however, under conditions of hyperirritability of the brain; in convulsive states the amounts of ammonia may be as high as 0.45 mg. per 100 gm. tissue. It is found in the brains of hibernating animals (115) and increases in amount with the increased brain activity after awakening of the animals. The adenylic acid in the brain decreases under such conditions. Normal blood ammonia is decreased during ether anaesthesia (116).

As for other bases, it is now reported that spermine and spermidine are present in brain (224).

*Phosphocreatine.*—The phosphocreatine of the brain is not affected by narcotics (64). It is present in the retina (117), which also contains creatine in amounts comparable with that in brain; adenylypyrophosphate is also present (see also 186).

*Acetylcholine.*—Owing to the physiological importance of this substance there has been a large amount of work recently carried out on the production and breakdown of acetylcholine in brain, and it will be impossible to deal adequately with the literature in the space allotted to this review. The subject has been reviewed previously by Bacq (118), Gaddum (119), Gerard (1), and Kahane & Levy (155).

Additional information has accumulated concerning the liberation of acetylcholine from nerve, etc. Acetylcholine (or its equivalent) is liberated when the diaphragmatic nerve and the vagus nerve are cut and the cut end immersed in Ringer solution (120). When isolated lungs are perfused with oxygenated Tyrode solution at pH 6.5 to 7.0 and the pulmonary vagus is electrically stimulated, there is a diffusion of acetylcholine into solution (121). A perfusate from guinea-pig heart during anaphylactic shock contains acetylcholine (122). The ester is formed in the pancreas (123). It is liberated into the venous blood of stomach, intestine or pancreas on stimulation of the splanchnic nerve (124) and it is liberated also by the superior cervical sympathetic ganglion (125). It is produced from intact intestine (126) and from human sympathetic ganglia (127). According to Loewi & Hellauer (128) acetylcholine is present in all efferent nerves. Preganglionic fibres contain six times as much of the ester as postganglionic fibres. Purely sensory nerves do not contain the ester. The isolated brain and spinal cord of the toad, immersed in eserine solution, liberate acetylcholine on direct stimulation. Isolated medulla

liberates the ester on central stimulation of the vagi (129). Acetylcholine liberation from sympathetic ganglia has been studied by Brown & Feldberg (130). Brown's results (133) support the view that neuromuscular transmission in amphibian and mammalian muscle is effected by liberation of acetylcholine at nerve endings.

It has been shown by Quastel, Tennenbaum & Wheatley (131) that a choline ester is synthesised by brain-cortex slices in an eserine medium, aerobic condition and the presence of glucose, lactate or pyruvate being necessary. The presence of glutamate and  $\alpha$ -glycerophosphate also brings about choline ester synthesis but the effect is much less marked than with glucose; the presence of succinate, although securing a large oxygen uptake by the brain tissue, has no effect on the synthesis. Their results indicate a link between glucose metabolism and the synthesis of the choline ester. No synthesis takes place in kidney, liver, spleen, or testis, but muscle (diaphragm) shows some activity. The ester has the pharmacological properties of acetylcholine, and that it is identical with this ester is made practically certain by the isolation of acetylcholine from brain tissue by Stedman & Stedman (132). The latter workers (134) claim that acetoacetic acid is a precursor of acetylcholine in brain, but Mann, Tennenbaum & Quastel (14, 135) are opposed to this conclusion on the grounds that under optimal respiratory conditions acetoacetic acid causes no synthesis of acetylcholine whereas, under similar conditions, glucose, lactate or pyruvate are highly effective. Any consideration concerning acetylcholine synthesis must take into account the fact that an acetylcholine complex (pharmacologically inactive) is present in brain which breaks down under a variety of conditions to form free acetylcholine. The existence of such a complex has been noted by Corteggiani *et al.* (136, 138, see also 228) who have shown that the complex breaks down to free acetylcholine on heating. Mann *et al.* (14) conclude that the complex is broken down by denaturing agencies (heat, chloroform, acid) to free acetylcholine, that it is synthesised in brain under optimal respiratory conditions and that it is a precursor of the acetylcholine formed in brain (see also 141). Pinotti (139) finds that much acetylcholine is present in an inactive state in cholinergic fibres, the vagus in particular. Chang (140) notes a similar phenomenon in connection with placenta and finds evidence of the liberation of acetylcholine into the cerebrospinal fluid by the afferent vagus. Kahane & Levy (141) discuss the effects of anomalies of extraction on the appearance of free and combined acetylcholine. Trethewie (142) con-

cludes that the "combined" acetylcholine is an association of acetylcholine with cell debris, etc., but it is clear that since acetylcholine is not freely diffusible in the cell it must be combined in some fairly well-defined way. Experiment (14) has shown that acetylcholine is not adsorbed by various brain components in amounts which would account for the amounts of the acetylcholine complex synthesised in brain, and it is suggested (14) that it exists in brain as a specific protein complex (see also 150). Since the demonstration of synthesis of acetylcholine in tissues *in vitro* it is evident that such tissues should be examined under optimal physiological condition to obtain true estimates of the formation in them of acetylcholine. MacIntosh (143) finds that glucose influences markedly the rate of formation of acetylcholine in the cat's superior cervical ganglion perfused with an eserine-Locke solution. Dikshit (144) has shown that acetylcholine formation takes place in brain cortex, basal ganglia, spinal cord, sympathetic ganglia, auricle, urinary bladder, gastrointestinal tract and pancreas and concludes that nerve plexuses are chiefly responsible for the synthesis. Mansfeld *et al.* (62) find that no decrease in acetylcholine formation takes place in the isolated brain of narcotised animals, but their experiments are open to the criticism that the narcotic diffuses from the tissue into the surrounding medium. Acetylcholine in the nerve ganglia of molluscs has been reported (145). Fluoride inhibits acetylcholine synthesis (146); cobra venom breaks down combined acetylcholine (147) whilst, apparently, tetanus toxin increases and strychnine decreases the acetylcholine content of the central nervous system (148). Miller (149) concludes that a response to eserine may be evoked locally from the cerebral cortex. Deutsch & Raper have shown that acetylcholine increases the respiration of submaxillary gland (154).

#### HYDROLYTIC ENZYMES IN THE NERVOUS SYSTEM

*Cholinesterase*.—This physiologically very important enzyme has been extensively studied. Nachmansohn (156) finds that the cholinesterase activity of the grey matter of spinal cord is 10 to 20 times greater than that of white matter, and that the enzyme is concentrated in tissues containing nerve endings. In the nervous system of crustaceans there is a higher concentration of the enzyme in the ganglion cells than in nerve fibres. From studies of cholinesterase activity Nachmansohn considers (157) that acetylcholine may play the

same rôle in the central synapses as in sympathetic ganglia and neuromuscular functions (see also 158). Bacq & Nachmansohn (159) point out that the hydrolysis of acetylcholine by the brain ganglia of molluscs is more than ten times more rapid than that by muscle. Evidence has been provided from studies of the effects of nerve degeneration (160) that the enzyme is localised at a high concentration in the end plates of muscle (see also 167, 176) and its distribution in the nervous system has been studied by Nachmansohn (161). The enzyme accumulates at the synapses and there is a parallelism in the tissues of chicken embryos between the development of synapses and the concentration of the enzyme (161). Brücke (162) has reported on cholinesterase in sympathetic ganglia and shows (163) that a diminution of the esterase takes place in degenerated preganglionic fibres. Martini & Torda (164, 165, 166) have shown that denervation of muscle leads to a gradual drop in cholinesterase activity and have studied the fall of esterase activity after section of spinal cord and destruction of cerebellum.

According to Glick (168), the maximum cholinesterase activity of the superior cervical ganglion of the cat is equivalent to the splitting of 0.1  $\mu$ g. acetylcholine per mg. fresh tissue per sec. at pH 7.4 and 38°. He concludes that if there is to be removal of acetylcholine during the 2 msec. refractory period of the ganglion there must be localisation of enzyme and substrate in the ganglion. The dissociation constant of the reaction between cholinesterase and acetylcholine is 0.001. From studies on the kinetics of cholinesterase *in vivo* Clark & Raventós (169) doubt the existence in muscle of esterase activity sufficient to hydrolyse within a few milliseconds the acetylcholine liberated at motor nerve endings. Below a concentration of 0.001 per cent the velocity of hydrolysis of acetylcholine by the enzyme varies with the concentration of ester (170). The specificity of cholinesterase has been studied by Easson & Stedman (171), Glick (172), Kahane & Levy (173) and micromethods for its estimation have been elaborated (174, 175). As found by Roepke (177), Ziff, Jahn & Renshaw (178) show that the relative affinities of various compounds for cholinesterase bear no relationship to their physiological activities, and the possibilities of ionic exchange (by adsorption) of choline derivatives on a protein surface with liberation of free acetylcholine are discussed (150). Anti-cholinesterases have been studied by Kahane & Levy (179), Schweitzer *et al.* (180), Bernheim & Bernheim (181), Kahn & Surles (182). Morphine and its derivatives in-



hibit cholinesterase (181, 182) and also reversibly inhibit the response of eserinated leech muscle to acetylcholine (183).

*Phosphatases.*—Cedrangolo & Ruffo (184) have investigated the acid and alkaline phosphatases of brain. According to Fleischhacker (190) alkaline phosphatase is united firmly to brain, extracts of which do not show the maximum activity of the tissue. Hexosediphosphate is hydrolysed to a greater extent by white than by grey matter and inosinic acid is hydrolysed. Motor cortex has the highest phosphatase activity. Reis (247) has demonstrated the existence in nerve tissue of a specific phosphatase, 5-nucleotidase, which breaks down adenylic acid and inosinic acid. It is found in grey and white matter, in peripheral nerve, and to the greatest extent in retina. Magnesium activation of brain phosphatases has been shown to be increased on purification of the tissue extracts (185).

*Other hydrolytic enzymes.*—Blum *et al.* (187) could find only dipeptidase in a search for proteolytic enzymes in brain (see also 249). Michel *et al.* (188) report a slow hydrolysis of acetanilide by brain in contrast to the high activities of liver or kidney. Brain, in contrast to liver, will not hydrolyse homatropine and atropine (189).

### CORTICAL POTENTIALS

The volume of work on this subject is rapidly increasing and efforts are being made to link the phenomena with changes of brain and nerve metabolism. The new technique of investigating cortical potentials of the brain *in vivo* or with the isolated brain promises to lead to interesting developments in our knowledge of the metabolism of the nervous system.

A review of factors controlling brain potentials has been published by Gerard (191). It is now well known that carbohydrate metabolism, in particular glucose oxidation, plays a dominant rôle in brain respiration and it has become evident that cortical potentials are greatly influenced by glucose metabolism in brain. The results of Rubin *et al.* (192) support the view that a quantitative relationship exists between carbohydrate metabolism and the alpha frequency. Hoagland *et al.* (193) show that in insulin hypoglycaemia there is a marked change in the nature of the cortical potentials and that electrical activity ceases if the hypoglycaemia is prolonged. Gibbs, Gibbs & Lennox (194), who have investigated the cortical potentials in epilepsy (see also 255), show that luminal slows the rate of cortical waves,

that hyperglycaemia prevents *petit mal* waves whilst hypoglycaemia causes them to become almost continuous.

Beecher, McDonough & Forbes (195) find that a fall of blood pressure in anaesthetised cats is followed by cortical changes indistinguishable from those caused by an increase in anaesthesia. The alteration in the amplitude of brain waves due to fall of blood pressure and resultant anoxaemia has been studied by Dow (196), and the changes of brain potentials due to abrupt and complete stoppage of blood flow and its release have been investigated by Sugar & Gerard (197). The abolition of brain potentials in anoxaemia is shown by Davis, Davis & Thompson (198) and Hoagland (199).

Maddock, Hawkins & Holmes (200) have shown that with eviscerated hepatectomised animals, the frequency of cortical potentials becomes lower with fall of blood sugar and that at low blood-sugar levels the waves may disappear. The administration of glucose results in the restoration of the original rhythm after a short interval, depending apparently on the severity and duration of the preceding hypoglycaemia and on the dose of glucose administered. Recovery is also brought about by the administration of mannose and maltose but not by that of hexosediphosphate, galactose, fructose, succinate, fumarate, pyruvate or glutamate. They comment upon the possible connection between cortical activity and acetylcholine formation which is known to depend greatly on glucose metabolism (14, 131).

The relationship between brain potentials and physiological variables has received attention (201) e.g., the effects of pH (202) and of changes in potassium, calcium and magnesium concentrations (203, 204, 213). The stimulating action of acetylcholine on cortical potentials has been demonstrated by Bonnet & Bremer (204) and work has been done on the action of strychnine and acetylcholine on the electrical activity of the nervous system of crustacea (205). A diminution of frequency of cortical potentials takes place after alcohol and ether administration (206). The influence of glucose, potassium, acetylcholine, and of various drugs on the electrical activity of retina has been studied by Therman (207). Cerebral activity during sleep has been investigated by Bremer (208) and by Blake & Gerard (209).

#### PERMEABILITY OF NERVE TISSUE, AND THE BLOOD-BRAIN BARRIER

Questions of permeability of the nerve cell cannot be neglected in any work on brain and nerve metabolism and more information on this subject is wanted. The effects of venom, morphine, picrotoxin

(210) and of tetanus toxin (211) in influencing the diffusion of potassium ions have been investigated. Tetanus toxin increases diffusion of the potassium ion to a greater extent than strychnine or electrical stimulation. According to vanHeuverswyn (253), permeability to ions varies with pH, there being significant alterations in permeability to potassium ions between pH 6 and 8. The effects of anoxaemia on permeability are of particular importance. The concentration of blood potassium changes as a result of partial asphyxia (212, see also 213) and it is of interest in this connection that blood-serum potassium is reported to increase 100 per cent during the convulsive attacks induced by cardiazole (214).

Friedemann (215) finds that the cerebral capillaries are more permeable to basic than to acid dyes and that the permeability is greatly determined by the electrical charge of the dye. Permeability is increased by epinephrine. Staphylococcus toxin is unable to pass the blood-brain barrier. The permeability to nitrate is slightly increased in cases of experimental poliomyelitis (216). Diffusion of alcohol into the cerebrospinal fluid has been investigated by Goldberg (217) and Riklin (254). Alcohol increases penetration of bismuth into the brain and spinal cord (218). Urea passes quickly into the brain but only slowly into the cerebrospinal fluid (219). Alcohol increases arsenic retention (or access) to the brain (220). Trypanocidally active arsenic appears in the cerebrospinal fluid after tryparsamide administration, but little or none after injection of trivalent arsenic compounds (221).

#### VARIOUS CHEMICAL CONSTITUENTS

*Salts, etc., in the nervous system.*—Tupikova & Gerard (222) have carried out analyses of the salt content of neural structures; these analyses demonstrate the high potassium content of central regions especially of grey masses. Cerebellum has the highest potassium content. Magnesium is decreased in vitamin-B<sub>1</sub> deficiency (251). Minerals (iron, calcium, sodium, magnesium) in the brain have been investigated under normal and pathological conditions by Alexander & Myerson (223); their amounts in the grey matter are greater than in the white. Normal white matter is richer in phosphorus (see also Tingey, 246, who has estimated the iron, copper, and manganese contents of various parts of the human brain).

*Phospholipids, etc.*—McConnell & Sinclair (229) find that brain incorporates elaidic acid into cephalins and lecithins more slowly than

muscle and liver. Using radioactive phosphorus as indicator, it has been found that phospholipids are more slowly deposited or synthesised in brain than in other organs (230, see also 233). Changus, Chaikoff & Ruben (231) find, using the same indicator, that there is a slow deposition of radioactive phospholipid in brain for as long as 200 hours after injection of the labelled phosphate, the rates being identical in fed and fasted animals. The loss from brain is very slow. Phospholipids in peripheral nervous tissue are reduced in diabetes, neutral fat being increased (232). The amino acid content of various mammalian brain proteins has been investigated by Block (234), who has shown the existence of sex differences in the amino acid composition (lysine:arginine) of neuroprotein. Randall (235) gives data on the composition (phospholipid, cholesterol, nitrogenous constituents, etc.) of various parts of the brain.

*Ascorbic acid and lactoflavin.*—Wortis *et al.* (236) find the R.Q. of scorbutic guinea-pig brain to be unity and such brain respire at the same rate as the normal. The ascorbic acid content of brain falls during hibernation (237). The amounts in the brain of guinea pigs (33 to 155  $\mu\text{g. per gr.}$ ) (238), hens (239) and of monkeys (80  $\mu\text{g. per gr.}$ ) (240) have been estimated and a combined form of ascorbic acid (ascorbigen) in guinea-pig brain reported (241). Lactoflavin is present in brain: 2.2  $\mu\text{g. per gm.}$  in the grey matter of sheep brain and 1  $\mu\text{g. per gm.}$  in the white matter (242, see also 248). Neurological manifestations of vitamin B<sub>2</sub> deficiency are described by Zimmerman *et al.* (243).

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WALES

## THE ALKALOIDS\*

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The various alkaloid groups considered in this section have been arranged as far as possible in the same order as in the preceding review of the field.<sup>1</sup> It is intended that all types in which notable advances have been made in the biennium shall be mentioned, if only briefly, but it is obviously not practical to include the numerous investigations that have resulted solely in the isolation of new alkaloids, however important these discoveries may be as the starting point of further studies.

*Alkamine esters.*—The alkamine retronecine, the basic fragment from the hydrolysis of senecionine, retrorsine, jacobine, squalidine, trichodesmine, and seneciphylline, is converted by hydrogenation to retronecanol,  $C_8H_{15}NO$ . On successive dehydration and hydrogenation, retronecanol passes through heliotridene,  $C_8H_{13}N$ , to heliotridane,  $C_8H_{15}N$  (1, 2a), identical with the products previously obtained from heliotridine, the basic portion of heliotrine and lasiocarpine. Similarly, by chlorination and reduction, platynecine, *ex* platyphylline, is transformed directly to heliotridane. These reactions establish a close relationship between the *Senecio* alkaloids (Compositae) and those of the Boraginaceae, that is further evident in the identification of trichodesmidine (3) with retronecine (2a). The heliotridane skeleton is tentatively represented as in I (2b).



I

In view of the size of the genus *Senecio* (about 1250 species), it is to be expected that new alkaloids are reported. From *Senecio jacobaea*, jacobine and jaconine have been isolated (4), and from *S. isatideus*, a new base, isatidine,  $C_{18}H_{25}NO_7$ , which on hydrolysis yields isatinecine,  $C_8H_{13}NO_3$  and isatinecic acid,  $C_{10}H_{16}O_6$  (5).

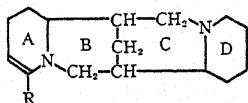
The already numerous tropine derivatives have been increased by new alkaloids from the Australian plant *Duboisia myoporoides*.

\* Received December 28, 1938.

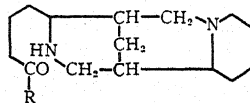
<sup>1</sup> *Ann. Rev. Biochem.*, 6, 513 (1937).

This has yielded, in addition to hyoscyne, tigloidine or tiglylpseudo-tropëine, valeroidine, which is isovaleryldihydroxytropene, and poroidine and isoporoidine, identified respectively as isovaleryl*nortropëine* and *d*- $\alpha$ -methylbutyryl*nortropëine* (6).

*Lupinane-sparteine alkaloids*.—New evidence substantiating the nature and location of the lactam carbonyl group of lupanine has been developed by Winterfeld (7) in the study of the action of organo-magnesium halides on lupanine. The tertiary alcohols first formed cannot be isolated, but lose water with great ease, yielding R-anhydro-lupanol (R-dehydrosparteines), formula II. Saturation of the new double bond results in R-substituted sparteines. With respect to increasing action on the frog's heart, the substituted sparteines are arranged in the order methyl, ethyl, phenyl, anisyl (8).



II. Dehydrosparteine structure  
(R = alkyl or aryl substituent)



IIIa. Phenylsparteone (R = C<sub>6</sub>H<sub>5</sub>)  
 IIIb. Sparteinic acid (R = OH)  
 IIIc. N-Benzoyl- $\omega$ -phenylsparteone  
 (R = C<sub>6</sub>H<sub>5</sub>, >NH = >N·CO·C<sub>6</sub>H<sub>5</sub>)

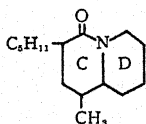
Phenyldehydrosparteine (II, R = C<sub>6</sub>H<sub>5</sub>) suffers hydrolytic scission in the presence of mineral acids to give an imino ketone, phenylsparteone (III a). By catalytic reduction at the ketone group, phenylsparteone (III a) is converted, probably through an intermediate  $\omega$ -phenylsparteol, to phenylsparteane, in which the presence of the imino group can be demonstrated by acylation.

In further proof of the presence of the lactam group in lupanine the piperidone ring A in this alkaloid can be opened with fuming hydrochloric acid, to yield an imino acid (III b), whose ethyl ester is identical with sparteinic acid ethyl ester (9).

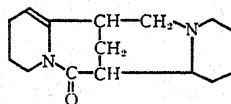
The well-known difficulty encountered in the stepwise oxidative degradation of sparteine and lupanine to recognizable large fragments has been to some extent overcome through the use of N-benzoyl- $\omega$ -phenylsparteone (III c) as starting material (10). In addition to succinic and *d*-aminovaleric acids, a diketone, believed to be 3,5-diketo-octahydropyridocoline, was obtained. The *d*-aminovaleric acid must arise from a portion of the molecule containing a chain of four secondary carbon atoms directly attached to nitrogen. Such a struc-

ture exists only in ring A of lupanine, or in ring D if the latter ring is of the piperidine and not the methylpyrrolidine type. Since ring A is already opened in phenylsparteone (III *a*), the appearance of *d*-aminovaleric acid may be considered as additional evidence of the six-membered nature of ring D. This hitherto uncertain structural entity appears also in the form of N-methylpiperidone after Clemmensen reduction of the oxidation mixture (10). The above-mentioned diketo-octahydropyridocoline probably represents rings C and D of lupanine. These results confirm the structure already deduced from the degradation of  $\alpha$ -didehydrosparteine, and from Clemo's synthesis of *dl*-oxysparteine.<sup>2</sup>

Previous attempts to degrade sparteine by Hofmann's method have been complicated by the participation of both nitrogen atoms in the reaction. To confine the degradation to one nitrogen, Späth (11*a*) has used oxysparteine, in which the basicity of the nitrogen atom common to rings C and D has been decreased by the amide structure. The end product of degradation and hydrogenation, hexahydro-hemioxysparteylene, probably has the structure IV. Attempts to convert it by dehydrogenation to derivatives of 8-oxo-*ps*-quinolizine-(8) were unsuccessful. Aphylline, the dihydro derivative of aphyllidine (V) is an isomer of oxysparteine; it can be reduced to *d*-sparteine (pachycarpine) (11*b*). In view of these relationships it is a striking coincidence that oxysparteine and aphylline have equal and opposite rotatory power, although they differ in melting points; the possibility of optical isomerism should be investigated.



IV. Hexahydro-hemioxysparteylene



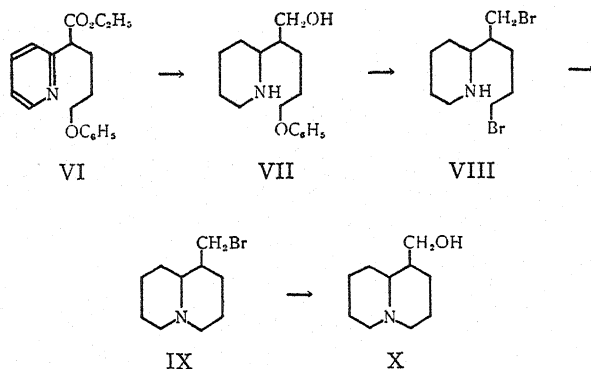
V. Aphyllidine

The degradation products reported from cytisine (12), namely 2,4-dimethyl-8-oxo-*ps*-quinolizine-(8) and 3,5-dimethyl-2-*n*-propylpyridine have been synthesized (13).

A complete synthesis of *l*-lupinine has been accomplished by Clemo (14). Ethyl 2-pyridylacetate was condensed with  $\gamma$ -phenoxy-*n*-propyl bromide to VI, which, on successive catalytic and Bouveault

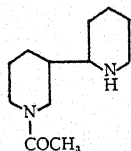
<sup>2</sup> Cf. *Ann. Rev. Biochem.*, 6, 515 (1937).

reduction, gave the carbinol VII. The action of fuming hydrobromic acid resulted mainly in VIII, which cyclized easily to IX, a mixture of two racemic bases. These were separated and hydrolyzed, yielding *dl*-isolupinine and *dl*-lupinine (X); the latter base was resolved with tartaric acid.

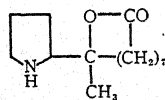


Synthesis of *l*-lupinine

*Pyrrolidine and piperidine groups.*—In *Ammodendron conollyi*, Orekhov (15) has found, together with *d*-sparteine (pachycarpine), the new base ammodendrine,  $C_{12}H_{20}N_2O$ . This alkaloid contains one unsaturated linkage, whose location is not certain, but dihydroammodendrine was shown to have the structure XI. Ammodendrine is of interest as one of the few known N-acetyl alkaloids [cf. colchicine and N-acetylmescaline (16)], and further, because of its occurrence in one of the Leguminosae, accompanied by the unrelated *d*-sparteine.



XI. Dihydroammodendrine



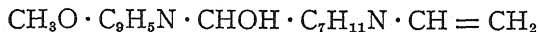
XII. Carpaine

The last point has its parallel in the appearance of the sparteine types aphylline and aphyllidine with anabesine in *Anabasis aphylla* (Chenopodiaceae).

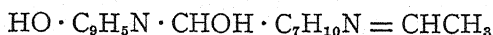
The discovery that carpaine contains a C-methyl group puts out of consideration the  $\beta$ -lactone formula previously proposed for the

alkaloid,<sup>3</sup> since such a structure, if it contained a C-methyl, could not yield azelaic acid on oxidation. The most probable structure, XII, accounts for the fact that the product of hydrolysis, carpamic acid, cannot be converted back to carpaine, and for the presence of a tertiary alcoholic group in carpamic acid. Synthetic work with carpamic acid as its objective is in progress (17).

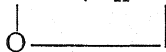
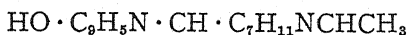
*Quinoline group; cinchona alkaloids.*—Recent studies in this series have been concerned largely with the search for derivatives that might possess antiplasmodial or pneumococcal properties. The demethylation and rearrangements of quinine, cinchonine, quinidine, and cinchonidine have been investigated by Henry (18) and Solomon (19). Apoquinine has been obtained in crystalline form, and its formula confirmed. In the demethylation reaction an isomer, isoapoquinine, is formed as well as hydroxydihydroapoquinine. Confirmatory evidence is advanced that the quinine—apoquinine change involves the rearrangement XIII to XIV, as suggested by Suszko (19 *a*), and that the other numerous products attained through the demethylation of quinine and quinidine are best accounted for by the type formulas XV and XVI.



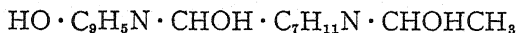
XIII



XIV



XV



XVI

Hydrogenation of type XIV regenerates the asymmetry at C-3, the point of attachment of the unsaturated group to the quinuclidine system, and yields the first known diastereoisomers differing only at this point. The addition of hydrogen chloride at the vinyl group

<sup>3</sup> Cf. *Ann. Rev. Biochem.*, 4, 498 (1935).



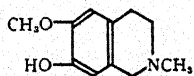
of type XIII and the antimalarial value of the products have been studied by Goodson (20). The antiplasmodial action of a number of cinchona derivatives and related synthetic substances is reported by King (21). Of the numerous apocupreine [apoquinine, cf. Henry (22)] derivatives studied by Butler (23),  $\alpha$ -methyl- $\beta$ -hydroxyethyl-apocupreine was found to possess high pneumococcal activity and relatively low eye-damaging effect.

Two new syntheses of quinuclidine [bicyclo(2,2,2)aza-1-octane] make this hitherto rare material more accessible. Clemo (24) utilizes a Dieckmann condensation of ethyl piperidine-1-acetate-4-carboxylate with subsequent hydrolysis and decarboxylation to obtain 2-ketoquinuclidine, which can be reduced to quinuclidine. Prelog (25) converts tetrahydropyran-4-acetic ester to 4- $\beta$ -hydroxyethyltetrahydropyran. On vigorous treatment with hydrobromic acid this passes into 3-(2-bromoethyl)-1,5-dibromopentane, which reacts with ammonia to give quinuclidine.

*Tetrahydroisoquinoline group.*—It is many years since a new opium alkaloid has been reported. Wrede (26) has isolated from the seed-capsules of *Papaver somniferum* the phenolic base narcotine, which is 8-demethylnarcotine. It exhibits none of the antiscorbutic action claimed by Rygh (26a) for demethylated narcotine. King reports an unusual course of degradation of the  $\alpha$ - and  $\beta$ -hydroxylaudanosines (27).

*Salsola richteri* is found to contain salsolidine,  $C_{12}H_{17}NO_2$ , and salsamine in addition to salsoline. Salsolidine is the methyl ether of *l*-salsoline (28). Salsolidine was synthesized by hydrogenation of 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline. By N-methylation of *d*- and *l*-salsolidine, the corresponding forms of carnegine were obtained (29).

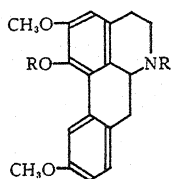
The alkaloid corypalline, from *Corydalis pallida* and *C. aurea*, has been shown to have the structure XVII, and has been synthesized. The preferred structure for corluminine<sup>4</sup> has now been proved to be correct (30). The studies of Manske on fumariaceous plants continue to produce numbers of new alkaloids (31).



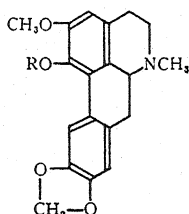
XVII. Corypalline

<sup>4</sup> Cf. *Ann. Rev. Biochem.*, 6, 521, 522 (1937), Formula XXIV.

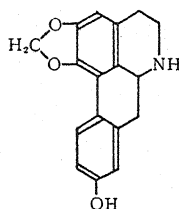
*Aporphine group.*—The synthesis of *l*-N-methyltuduranine methyl ether (XVIII *a*) by Goto (32) places the solution of the tuduranine structural problem in immediate prospect. The 3,5,6 positions of the peripheral substituents is assured, and the 5 position for the hydroxyl group seems excluded by the non-identity of the degradation product of the synthetic compound XVIII *b* with that from N-ethyltuduranine ethyl ether. The decision between positions 3 and 6 for the hydroxyl group must await the synthesis and degradation of the corresponding ethyl derivatives.



XVIIIa. *l*-N-methyltuduranine methyl ether  
(R = CH<sub>3</sub>)  
XVIIIb. R = C<sub>2</sub>H<sub>5</sub>



XIXa. Domesticine  
(R = H)  
XIXb. Epidicentrine  
(R = CH<sub>3</sub>)



XX. Anolobine

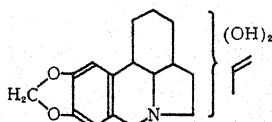
Conclusive proof of the structure of domesticine (XIX *a*) has been obtained through the synthetic work of Kitasato & Shishido (33, 34). Racemic epidicentrine XIX *b* was prepared by 6',8-cyclization of diazotized 6'-amino-N-methyl-1-piperonyl-6,7-dimethoxy-tetrahydroisoquinoline, and the dextro form was found to be identical with domesticine methyl ether. *d*-Epidicentrine is also the nantenine, or domestine of Takase and of Maniwa. Determination of the position of the domesticine phenolic hydroxyl group was made through the usual device of synthesizing the ethyl ether for direct comparison. The alternative, 6-ethyl derivative, was furthermore shown to be different from domesticine ethyl ether.

*Asimina triloba* Dunal, has yielded the new alkaloid anolobine, C<sub>17</sub>H<sub>17</sub>NO<sub>8</sub>, for which the structure XX is believed to be probable (35).<sup>5</sup>

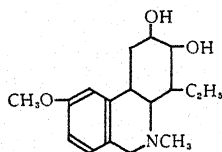
*Lycoris alkaloids.*—While the synthetic work of Kondo (36) has assured a phenanthridine nucleus in lycorine, failure to locate the

<sup>5</sup> It should be noted that the structural formula favored by the investigator does not agree with the empirical formula.

carboxyl group in 6,7-methylenedioxy-N-methylphenanthridone-carboxylic acid, *ex.* lycorine anhydromethine, has delayed determination of the position of the fourth ring. Kondo (37) concludes that this ring extends from nitrogen to carbon-1, advancing as evidence the products obtained from zinc dust distillation of reduced lycorine anhydromethine, namely phenanthridine, 1-methylphenanthridine, and 1-ethyl-6,7-methylenedioxyphenanthridine. The structure of lycorine, down to the location of the unsaturated linkage and of the two hydroxyl groups, is represented in formula XXI (37). The isomerism of the lycorine methiodides has been attributed to asymmetry at the quaternary nitrogen atom (38). From oxidative degradation of lycoramine (one of the minor alkaloids from *Lycoris radiata*) products have been obtained that lead to the proposal of XXII for lycoramine (39).



XXI. Lycorine

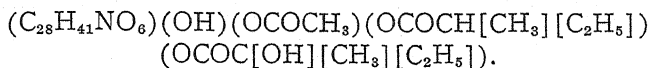


XXII. Lycoramine

*Veratrine group.*—Of the alkaloids of *Veratrum* species, jervine, pseudojervine, and rubijervine differ from germerine and protoveratrine in not being esters. Jervine,  $C_{26}H_{37}NO_3$  (40), is a secondary base containing one alcoholic hydroxyl group and one methylenedioxy group. Pseudojervine appears to be  $C_{33}H_{49}NO_8$  instead of  $C_{29}H_{43}NO_7$  as previously reported. It is a secondary base, with four hydroxyl groups. Rubijervine,  $C_{26}H_{43}NO_2$ , is a tertiary base, probably having two hydroxyl groups (41). Of the ester types, the formula of protoveratridine has been revised from  $C_{26}H_{45}NO_8$  to  $C_{31}H_{49}NO_9$ . Hydrolysis at the ester group results in germine,  $C_{26}H_{41}NO_8$ , and *l*-methylethylacetic acid. Protoveratridine is, however, not a true constituent of *V. album*, but is a product of partial hydrolysis of germerine, a new base of composition  $C_{36}H_{57}NO_{11}$ . The relationships appear as follows: Germerine  $C_{36}H_{57}NO_{11} \rightarrow$  methylethylglycollic acid  $(CH_3)(C_2H_5)C(OH)(CO_2H) +$  protoveratridine  $C_{31}H_{49}NO_9 \rightarrow$  *l*-methylethylacetic acid + germine  $C_{26}H_{41}NO_8$ . Germine appears to contain five non-phenolic hydroxyl

groups. Although it resembles cevine in many respects, the two are probably not identical.

Protoveratrine is  $C_{40}H_{63}NO_{14}$  instead of  $C_{32}H_{51}NO_{11}$ , and gives on hydrolysis, methanol, *l*-methylethylacetic acid, and methylethylglycollic acid, together with protoverine,  $C_{28}H_{45}NO_{10}$  (42). Its formula may be resolved into

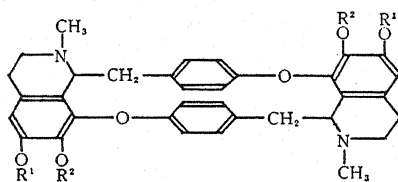


The degradation of cevine, the basic constituent of veratrine and cevadine, has been studied by Jacobs & Craig (43). The most significant products isolated from soda-lime distillation were *d*- $\beta$ -pipecoline, 2-ethyl-5-methylpiperidine, and a dicyclic base (44)  $C_{10}H_{19}N$  that appears to be closely related to the 2-ethyloctahydropyrrocoline of Clemo (45). Zinc dust distillation of cevine gives in addition to *d*- $\beta$ -pipecoline, *d*-*N*-methyl- $\beta$ -pipecoline and a base that is believed to be 2-ethyl-5-methylpyridine. It seems quite certain that no coniine was formed in these degradations (44). There is evidence that cevine-methine is an enolbetaine, but its degradation has not yet given identifiable products (46).

*Aconite alkaloids.*—The base atisine, apparently the sole alkaloidal constituent of atis root (*Aconitum heterophyllum* Wall.) is found by Lawson (47) to be  $C_{22}H_{33}NO_2$  rather than  $C_{22}H_{31}NO_2$  or  $C_{22}H_{35}NO_4$  as previously reported. The parent nucleus is probably pentacyclic,  $C_{20}H_{31}N$ , in contrast to that of aconitine, which Freudenberg (48) believes to consist of two five-membered and four six-membered condensed rings, probably a new alkaloid ring system not related to the cyclopentenophenanthrenes. Attempts to determine the nature of the hypothetical aconitine nucleus  $C_{19}H_{28}NH$  have resulted in a new, indifferent compound  $C_{19}H_{24}O$ , apparently a five-ring system with three non-benzenoid double bonds and one hydroxyl group. The oxidative attack on the aconite alkaloids has been pursued by Sugimoto (49) and Tamura (50) who depict the resolved-formula relationships of jesaconitine, aconine, oxinitine, oxinine, mesaconitine, and mesaconine, as well as other bases of the series.

From *Aconitum* residues Freudenberg has separated two new alkaloids, neoline  $C_{24}H_{41}NO_6$ , and napelline  $C_{22}H_{33}NO_3$ , together with traces of *l*-ephedrine and sparteine (51). Selenium dehydrogenation of napelline gives  $C_{17}H_{16}$ , apparently the same hydrocarbon that Lawson & Topps (47) obtained from atisine (52).

*Curare*.—Recent advances in the curare field have been made chiefly in determining the nature of pot curare and calabash curare. From the former, King (53) has obtained the previously known (Boehm) protocuridine, and an isomer, neoprotocuridine  $C_{36}H_{38}N_2O_6$ . The isomers are diphenolic, and yield isomeric dimethyl ether dimethiodides, hence the isomerism is not due to a different arrangement of methoxyls and hydroxyls. Moreover, the former, in contrast to the latter, is optically active. By partial degradation, neoprotocuridine was shown to be an internally compensated form of isochondrodendrine, for which the tentative formula XXIII is offered. The alkaloids of pot curare are therefore closely related to those of tube curare.<sup>6</sup>



XXIII. Neoprotocuridine ( $R^1 = H$ ,  $R^2 = OCH_3$  or vice versa)

The active principle of pot curare is a phenolic, quaternary base of high paralyzing power, whose constitution is still unknown (53).

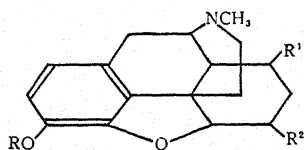
The alkaloids of calabash curare, now designated as calabash-curarines-I and -II, have been isolated by Wieland (54). The designation toxiferine, originally applied to the first of these bases, is now reserved for a much more active alkaloid from *Strychnos toxifera*, a species that, contrary to previous belief, does not seem to be a constituent of calabash curare. Little is known of the calabashcurarines, except that C-curarine-II appears to be the dihydro derivative of C-curarine-I; the latter is a quaternary base whose chloride has the formula  $C_{20}H_{23}ClN_2O$ . The quaternary base loses water with extreme ease to yield a dimolecular tertiary base  $C_{40}H_{42}N_4O$  that no longer shows curare action.

Carneiro reports the isolation of the very active curare alkaloids strychnoethaline  $C_{22}H_{27}NO_4$  and curaethaline  $C_{25}H_{30}NO_7$  (55). The isolation of a new base, erythroidine,  $C_{16}H_{19}NO_3$ , possessing curare-like action, from *Erythrina americana* Mill., is of interest. *Erythrina* species are not known to be an ingredient of curare (56).

<sup>6</sup> Cf. *Am. Rev. Biochem.*, 4, 510 (1935); 6, 523 (1937).

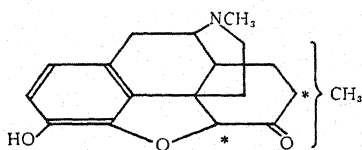
*Phenanthrene alkaloids.*—Systematic studies that have been conducted by Small, Mosettig, Eddy & Himmelsbach during the past nine years in the morphine group have had as object an understanding of the physiological significance of the various features of the morphine molecule rather than verification of the fairly well-established structure of this alkaloid. The results have been summarized in a recent publication that includes chemical, pharmacological, and clinical work on the morphine addiction problem (57). The comparison of thirty-four morphine derivatives with their phenol-ethers leads to the conclusion that the result of etherification is almost always an increase of convulsant action, and a decrease of all other morphine-like effects. The morphine alcoholic hydroxyl has been modified by etherification, or esterification, by steric or positional changes, or by substitution by other groups, ketonic, hydrogen, or basic groups. From a study of 86 pairs of such related derivatives, it is apparent that covering the alcoholic hydroxyl, or replacing it with hydrogen, halogen, or ketonic oxygen, increases most morphine effects, in contrast to replacement with basic substituents, as diethylamino or piperidino groups.

By the employment of special hydrogenation technique, the well-known tendency of the allyl ether types, such as pseudocodeine, to undergo reductive scission at the ether linkage can be largely suppressed. This procedure has made possible the preparation of numerous normal dihydro derivatives of such types in the morphine group, and might find application in other series of allyl ethers. It has permitted for the first time the evaluation of the pharmacological significance of groups occupying the 6- and 8- morphine nuclear positions, with elimination of any possible effect from steric differences (58). The 6-keto derivatives, dihydrocodeinone and dihydromorphinone (XXIV *a*), for example, are much more effective drugs than the 8-keto analogs (XXIV *b*).



XXIVa. Dihydromorphinone  
( $R=H$ ,  $R^1=H_2$ ,  $R^2=O$ )

XXIVb. Dihydroisomorphinone  
( $R=H$ ,  $R^1=O$ ,  $R^2=H_2$ )



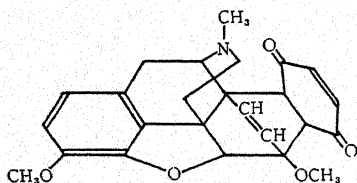
XXV. Methyl dihydromorphinone  
 $CH_3$  at one of starred positions

In similar comparative studies, estimation of the physiological significance of the oxygen bridge, and of alicyclic unsaturated linkages has been attempted (57).

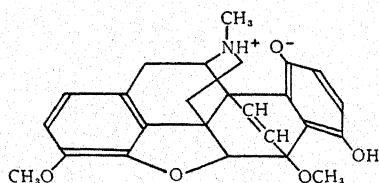
The reaction of organomagnesium halides with compounds of the pseudocodeine type results in the attachment of an organic radical to position 5 or 7 of the nucleus, with simultaneous scission of the ether bridge. By closure of the bridge and demethylation or reduction, nuclear organic radical substituted analogs of dihydromorphinone and dihydromorphine are obtained (59). Methyl dihydromorphinone (XXV) is the most interesting of these. It possesses greater analgesic action than morphine, and, in therapeutic doses, has almost no emetic or respiratory depressant action (60). The drug causes development of tolerance in man only very slowly and it does not appear to satisfy completely the craving of morphine addicts (57). It is interesting to note that the encephalogram resulting after administration of methyl dihydromorphinone to man is quite different from that produced by such drugs as morphine and heroin.

Through degradation of bromocodeine and synthesis of 1- and 2-bromo-3,4-dimethoxyphenanthrenes, Small (61) has demonstrated that bromination of morphine takes place in the 1- and not the 2-position as previously believed. This is of significance for the structure of pseudomorphine, postulated to be 1,1-dimorphine by Vongerichten (61*a*) from the fact that bromomorphine cannot be oxidized to a dimolecular product.

Sandermann (62) and Schöpf (63) have found that the diene system of thebaine condenses with *p*-benzoquinone, 1,4-naphthoquinone, and maleic anhydride. The success of this reaction with a methoxyl substituted diene is interesting, and suggests an extension to other enol ethers or enol esters. The adduct of thebaine with quinone, XXVI, undergoes rearrangement with great ease to thebainehydroquinone, XXVII, which behaves like a phenolbetaine. Thebainehydroquinone suffers demethylation and a deep-seated struc-



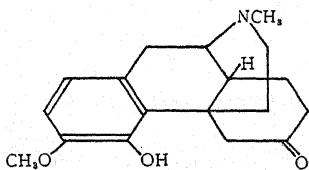
XXVI. Thebainequinone



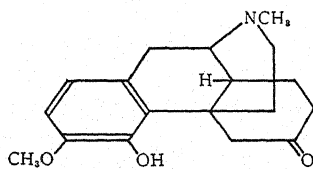
XXVII. Thebainehydroquinone

tural change with concentrated hydrochloric acid; the structure of the product, flavothebaon, is uncertain (63).

The reduction of thebaine under various conditions has been investigated by Small (64). Hydrogenation under neutral conditions (Wieland-Kotake procedure) results in tetrahydrothebaine, dihydrothebainol-6-methyl ether, and dihydrothebainone- $\Delta$ -5,6-methyl enolate. The last-named substance is obtained also by hydrogenation of thebainone methyl enolate, a rearrangement product of codeine methyl ether. Reduction of thebaine with sodium and alcohol (Freund procedure) gives phenolic dihydrothebaine, which can be hydrogenated to dihydrothebainone- $\Delta$ -6,7-methyl enolate. Hydrolysis of phenolic dihydrothebaine results in thebainone,  $\alpha$ -thebainone, and  $\beta$ -thebainone. From hydrogenation of  $\beta$ -thebainone, a  $\beta$ -dihydrothebainone (XXVIII) is obtained that is isomeric with the known dihydrothebainone (XXIX). The isomerism persists through the degradation intermediates down to  $\beta$ -thebenone, and evidence is advanced that it depends upon a configurational difference at C-14, the first well-substantiated example of such isomerism in the morphine group [cf. Schöpf (65)].



XXVIII.  $\beta$ -dihydrothebainone



XXIX. Dihydrothebainone

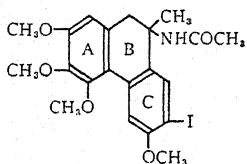
Discussion of the complicated series of isomeric methyl-dihydrothebaines resulting from interaction of thebaine and methylmagnesium iodide (66) may be deferred until more structural evidence is obtained.

Utilizing the Diels-Alder reaction, Fieser (67) has succeeded in synthesizing 3,4-dimethoxy-5,6,7,8,9,10,13,14-octahydrophenanthrene-13-carboxylic acid. Apparently only one of the two possible *dl*-forms was obtained. If the degradation of substances of the tetrahydro-desoxycodine methyl ether type can be carried to the carboxylic acid stage, a definite conclusion may be reached on the still unsatisfactory morphine ethanamine linkage question, although it is obvious that steric difficulties will be encountered.

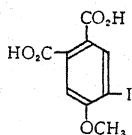
*Colchicine*.—Synthesis of the iodomethoxyphthalic acid obtained by



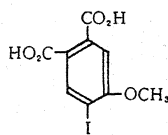
Windaus from oxidative degradation of N-acetyliodocolchinol methyl ether (XXX) shows it to be 5-iodo-4-methoxyphthalic acid (XXXI *a*) (68). This proves conclusively that the substituents in ring C of XXX occupy the 6 and 7 positions. Since, however, this phthalic acid derivative might have been produced equally well from an iodocolchinol ether with the alternative arrangement of substituents, as indicated in XXXI *b*, there still remains a little uncertainty in the colchicine formula.



XXX. N-acetyliodocolchinol methyl ether



*a*

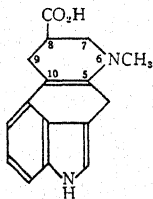


*b*

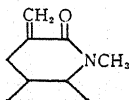
XXXI. 5-iodo-4-methoxyphthalic acid

The hydrogenation studies of Bursian (69) indicate that colchicine contains one resistant double bond in ring C. Hexahydrocolchicine gave by hydrolysis a primary amine, whose O-benzoyl-N-dimethyl derivative failed to show interesting physiological action.

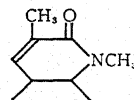
*Ergot alkaloids.*—Contrary to previous reports, Jacobs & Craig (70) observe that dihydrolysergic acid loses water on pyrolysis to yield a neutral, unsaturated product, probably of the structure represented in part-formula XXXIII *a* or XXXIII *b*.



XXXII. Lysergic acid



XXXIII *a*



XXXIII *b*

This fact, together with the results from a study of dissociation constants, indicates that lysergic acid is a  $\beta$ -amino acid (XXXII) and not an  $\alpha$ -amino acid as hitherto postulated.<sup>7</sup> Synthetic efforts have

<sup>7</sup> Cf. *Ann. Rev. Biochem.*, **6**, 530 (1937).

resulted in a compound, ergoline, containing the skeleton of dihydrolysergic acid (71). Ergoline, which gives color reactions almost identical with those of lysergic acid, is represented as the dihydro derivative of XXXII, with  $\text{CO}_2\text{H}$  and  $\text{CH}_3$  replaced by hydrogen. Comparison of the basic dissociation constants of dihydrolysergic acid and 6-methylergoline (decarboxydihydrolysergic acid) also points to the 8 position for the carboxyl group (72).

Stoll (73) has observed that when the ergot alkaloids are hydrolyzed with hydrazine hydrate instead of potassium hydroxide, the lysergic acid undergoes racemization and isomerization. The racemic isolysergic acid hydrazide so obtained suffers hydrolysis with simultaneous isomerization to yield rac. lysergic acid. By resolution of the amide resulting from interaction of rac. isolysergic acid azide and *l*-norephedrine, and subsequent hydrolysis, *d*-(ordinary) and *l*-lysergic acids are obtained. These results show that lysergic acid contains only one asymmetric center, in agreement with the formula (XXXII) of Jacobs & Craig. From the relative strength of the basic groups in lysergic and isolysergic acids, Craig (72) concludes that the double bond in these isomers is located at the 5,10 and 9,10 positions respectively.

Jacobs (74) now believes that the isobutyrylformic acid and the pyruvic acid resulting from the hydrolysis of the ergotoxine-ergotinine and ergotamine-ergotaminine pairs respectively, are not, as such, linked in the alkaloids, but are formed during the hydrolysis from the respective precursors,  $\alpha$ -hydroxyvaline  $(\text{CH}_3)_2\text{CHC}(\text{OH})(\text{NH}_2)(\text{CO}_2\text{H})$  and  $\alpha$ -hydroxyalanine  $\text{CH}_3\text{C}(\text{OH})(\text{NH}_2)(\text{CO}_2\text{H})$ . Formulas showing the probable mode of linkage of the constituents of these alkaloid pairs are advanced (72, 74).

Further study of ergosine and ergosinine (75) shows the correct formula to be  $\text{C}_{30}\text{H}_{37}\text{N}_5\text{O}_5$ . The pair are apparently condensation products of lysergic acid and isolysergic acid with *d*-proline, *l*-leucine, and pyruvic acid ( $\alpha$ -hydroxyalanine?, see above).

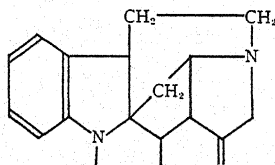
A new ergot pair, isomeric with ergotoxine and ergotinine has been found by Stoll (76). As with the other known pairs, the levo form, ergocristine, is highly active, the dextro form, ergocristinine is less active. Ergocristine, in molecular combination with ergosinine, is a constituent of Spanish and Portuguese ergot. The so-called ergoclavine seems to consist of similar molecular compounds of ergosinine with ergosine, or ergotamine with ergosinine (75, 76, 77, 78).

One of the most interesting developments in ergot chemistry is the

partial synthesis by Stoll (79) of ergometrine (ergobasine, etc.)<sup>8</sup> and its isomer, ergometrinine. This was accomplished by condensation of *d*-2-aminopropanol-1 with rac. isolysergic acid azide. The mixture of isomeric isolysergicisopropanolamides (ergometrinines) was rearranged with acid to the corresponding lysergic amides, which could be separated as tartrates into *d*-lysergic-*d*-isopropanolamide, or ergometrine, and *l*-lysergic-*d*-isopropanolamide. The antipode of ergometrine, *l*-lysergic-*l*-isopropanolamide, showed almost no ergot action. *d*-Isolysergic-*d*-isopropanolamide was identical with ergometrinine.

*Strychnine group*.—Robinson (80) finds the strychnine part-formulas suggested by Kotake<sup>9</sup> quite unacceptable. The isolation of tryptamine from potassium hydroxide fusion of strychnine and certain derivatives does not constitute any objection to the Leuchs formula, or to either of Robinson's proposals, for these all contain the tryptamine skeleton. Indeed, in previous publications Robinson has emphasized the significance of the tryptophan skeleton in the strychnine structural formula. Kotake's formulation, especially the transposition of the functions of N(a) and N(b), fails to account for some of the best established facts of strychnine chemistry.

The novel feature of a tentative proposal by Robinson (80, 81) is shown in part-formula XXXIV.



XXXIV

The fact that this structure does not contain a completed carbazole nucleus does not constitute any great objection, and the formula has the advantage of explaining most easily the monobromination of diketonucidine, the ready formation of tryptamine discussed above, and the blocked hydroaromatic nature of the hydroindole nucleus.

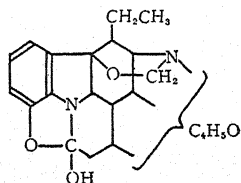
Space restriction permits only passing mention of several interesting attempts to synthesize degradation products or characteristic fragments of the strychnine system (45, 80, 82).

<sup>8</sup> Cf. *Ann. Rev. Biochem.*, 6, 529 (1937).

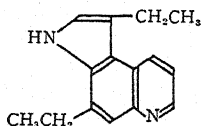
<sup>9</sup> Cf. *Ann. Rev. Biochem.*, 6, 529 (1937).

Leuchs (83) has found that 9-hydroxystrychnine,<sup>10</sup> the pseudostrychnine of Warnat,<sup>11</sup> is formed readily by oxidation of strychnine in the presence of the copper-ammonium complex. Pseudostrychnine is probably not a true *Strychnos* alkaloid, but is formed during the manufacturing process. It undergoes reversible isomerization that apparently depends upon a difference in the position of the double bond, for the isomers give the same dihydro derivative (84). 9-Hydroxystrychnine reacts in two forms, as  $>\text{C}(\text{OH})-\text{N}<$  and  $>\text{C}=\text{O} \text{HN}<$  (85). 9-Hydroxybrucine can be prepared by a similar oxidation of brucine (86).

As a result of reduction studies, Wieland (87) has reached the conclusion that vomicine may not resemble strychnine as closely in fundamental structure as previously believed. This idea has been confirmed by the behavior of the products arising from the progressive oxidative and pyrolytic degradation of vomididine, and finds expression in the tentative vomicine formula XXXV, which is offered with many reservations (88).



XXXV. Vomicine



XXXVI. Vomipyrine

The base  $\text{C}_{16}\text{H}_{23}\text{N}_2\text{O}_2$ , resulting from oxidation of vomididine and subsequent pyrolysis, gives on selenium dehydrogenation three products, the most abundant of which is vomipyrine,  $\text{C}_{15}\text{H}_{16}\text{N}_2$  (88). Formula XXXVI was suggested as a possible structure for vomipyrine. Synthetic 7,11-diethyl-5,6-pyrroquinoline (XXXVI) showed remarkable similarity in some physical and chemical properties with vomipyrine, but was not identical with it (89).

<sup>10</sup> According to Robinson's numbering system [cf. *Ann. Rev. Biochem.*, **2**, 446 (1933)] this substance would be 2-hydroxystrychnine.

<sup>11</sup> Cf. *Ann. Rev. Biochem.*, **2**, 450 (1933)

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# CHEMICAL ASPECTS OF PHOTOSYNTHESIS

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This review is not, and makes no pretense to be, a collocation of all papers on this subject which have appeared in the past two years. It represents, instead, a somewhat critical account of all such published work as would appear to offer a significant contribution to the problem best summarized by the equation  $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{CH}_2\text{O} + \text{O}_2$ .

Papers dealing with plant physiology in a general way have been omitted, even where observations on photosynthetic activity have been recorded. On the other hand, papers in relation to the reduction of carbon dioxide in the dark, which appear to the reviewer to have a bearing on the subject of photosynthesis, have been included. In this way it is hoped that a broad treatment of the subject has been attained, without sacrifice of any pertinent contribution essential to a clear understanding of the present-day position of scientific thought and development in this field.<sup>1</sup>

## METHODS

Warburg's manometric method has been employed, as in former years, by a large number of investigators (10, 12, 13, 14, 19, 23, 40, 49, 59, 65, 67, 68, 83, 92). It has been common practice with this method to use carbonate-bicarbonate buffer mixtures as a suspending liquid for unicellular algae. In most cases these buffers are not injurious to the algae, but it has now been ascertained that at higher pH values there is a difference between the rate of photosynthesis in carbonate and in phosphate buffers, which is not due to the pH [Emerson & Green (13)]. In this respect it may be of significance that phosphate ions catalyze the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$  whereas bicarbonate ions do not (74). Before drawing conclusions from experiments made with carbonate buffers a control with another medium is therefore advisable.

A new spectrographic method for the determination of carbon dioxide has been developed (53). This method takes advantage of the fact that carbon dioxide gas absorbs strongly the infrared radiation from 4.2 to 4.3  $\mu$ . It is especially adapted to the study of car-

<sup>1</sup> Citations of prior papers mentioned in the text but not listed here are marked (1a) and will be found in *Ann. Rev. Biochem.*, 6, 535 (1937).



bon dioxide exchange in higher land plants and gives quantitative results over periods as short as twenty to thirty seconds. The sensitivity is very high at low concentrations of carbon dioxide but decreases with higher concentrations of this gas.

An extremely rapid method for detecting carbon dioxide or oxygen exchange in plant metabolism consists in bringing plant tissues or layers of cells in direct contact with a glass electrode or a platinum electrode respectively (7). This method is the only one which records the time course of the photosynthetic gas exchange during the first second of illumination. It shows unmistakable responses to light flashes of a duration of less than one-tenth of a second.

With the artificial production of radioactive carbon a quite unique possibility has been provided for tracing the chemical reactions of carbon dioxide in living cells (75).

#### PLANT MATERIAL USED IN THE STUDY OF PHOTOSYNTHESIS

Many important papers on photosynthesis have been published in which the object studied has been a higher plant or a part of such a plant (leaves) (9, 53). It becomes increasingly evident, however, that any analysis of the mechanism of carbon dioxide reduction can be successfully carried on only with plant material grown under known conditions and investigated in the laboratory. Artificial cultures of aquatic plants, especially of unicellular algae, first used by Warburg, are much more suitable and can be expected to give more reliable results (1a). For the purpose of measuring bacterial metabolism no one would think nowadays of collecting bacteria grown under natural conditions. Picked leaves, cut branches, collected algae as they happen to be found in nature, all display a strong photosynthetic activity. This is the reason why so many papers are published in which accurate data on the metabolic activities of such plants are laboriously compiled. But for the proper interpretation of data from plants grown in natural surroundings and investigated under "natural conditions" (31, 32, 85) we shall probably have to wait until the results gained with pure laboratory cultures enable us to disentangle the complexities of conditions which, though "natural," are certainly not the best for scientific research in this highly complex field. Obviously, this criticism does not apply to papers devoted to purely ecological studies (79).

The modern work on respiration and fermentation in living cells has revealed a certain uniformity in the catalysts involved in these

processes as well as in the "paths of reaction" along which they proceed. Nevertheless, there is an abundance of individual variation so far as the details of the reactions are concerned. In all probability the same is true in photosynthesis. We have no reason to doubt that the process of carbon dioxide reduction will be essentially the same in all green plants. Yet a number of controversies caused by apparently contradictory observations (62, 23, 27) are found in the literature. These can be explained by the difference in the plant material used. Among the unicellular algae the deviations in metabolic behavior are striking and sometimes decisive for the success of an experiment. It is known that respiration in *Chlorella* does not proceed in the same manner as in *Scenedesmus* or in *Stichococcus*. Now equally important differences have also been found for the photosynthetic activity of different algae (12, 23). Even more surprising is the fact that photosynthesis in closely related strains, such as *Chlorella pyrenoidosa* and *Chlorella vulgaris* (12) or *Scenedesmus obliquus*, *Scenedesmus spec. D3* (27) and *Scenedesmus nanus* (62) will respond quite differently for instance to anaërobic treatment, or to the influence of cyanide. The culture methods and the age of the cells are of no less importance than the strain of the plants. The photosynthetic activity of *Chlorella pyrenoidosa*, expressed by the ratio, photosynthesis  $O_2$ (c.mm.)/chlorophyll (gm.) is highest in young cultures and declines with age (40, 67). The low activity of aged cells was found to be due to a deficiency of nitrates in the medium. Most of the algae investigated grow abundantly in media containing glucose (66, 40, 42, 27). It is interesting that the crop in such cultures cultivated in daylight is large in spite of the anaërobic periods in the dark, when the unilluminated algae are allowed to settle on the bottom of the culture flasks. In the presence of glucose light is favorable for growth even in the absence of carbon dioxide (42).

Algae grown in sugar solution often have a respiratory quotient,  $CO_2:O_2$ , higher than 1, sometimes approaching 2, but averaging about 1.4 (27). This shift of the respiratory quotient is likely to affect the measurements of photosynthesis. A stimulating influence of auxins on the growth of *Chlorella* in cultures older than eight to ten days has been observed (69).

Attempts have been made during the last few years to use isolated chloroplasts instead of intact cells as photosynthetic material (34, 35, 41, 43). The results of the different authors confirm the

early observation of Beijerinck and of Molisch that illuminated chloroplasts will release small amounts of oxygen. Though the methods for isolation of the chloroplasts have been improved, no true photosynthesis, *i.e.*, reduction of carbon dioxide, has been reported.

#### REDUCTION OF CARBON DIOXIDE IN THE DARK

Because all efforts have failed to show that in green plants carbon dioxide is reduced by the activated chlorophyll molecule itself, attention has been directed toward some unknown catalysts which might convert carbon dioxide into  $(\text{CH}_2\text{O})_n$  when sensitized by activated chlorophyll. It is now definitely known that carbon dioxide can be reduced in the dark by certain bacteria in metabolic processes which, in principle, may occur in any living cell [(1a), page 602]. The unknown catalysts in photosynthetic plants and purple bacteria may possibly be related to those which enable colorless bacteria to utilize carbon dioxide. All metabolic processes involving a chemical reaction with carbonates are therefore of great interest. As Barker (5) has shown, bacteria exist which will build up butyric and caproic acids with the simultaneous formation of acetic acid and methane, from ethyl alcohol and calcium carbonate as the sole source of carbon in an otherwise inorganic medium. Less obvious is the utilization of carbon dioxide by the propionic acid bacteria, as no net decrease in carbon dioxide has been obtained. But Wood & Werkman (103, 104) think it probable that carbon dioxide plays a part in most fermentations performed by these bacteria. 0.00125 *M* fluoride inhibits the carbon dioxide utilization in the propionic-acid fermentation of glycerol, whereas cyanide and azide, which inhibit photosynthesis, are without effect.

Small amounts of carbon dioxide are indispensable for the growth of several types of bacteria (33) as well as for certain reducing reactions of *B. coli* and *B. prodigiosum* (39). The fermentation of pyruvate by *B. coli* is accelerated by the presence of carbon dioxide and the formation of succinic acid is increased about 50 per cent in this reaction (11).

Most important for the problem of carbon dioxide reduction in the dark are the results of Ruben, Hassid & Kamen (75), obtained with radioactive carbon employed as a means of labeling the carbon dioxide gas. The reactions involved could be followed by measuring radioactivity. In the absence of light, the leaves of barley plants, yeast cells and—to a much smaller extent—cell-free sap

from fresh leaves are able to introduce the carbon of carbon dioxide into organic material. The organic material was analyzed for the presence of radioactive carbon after it had been separated into carbohydrate, acid, and other fractions. Only a very small portion of the radioactive carbon dioxide "reduced" in the dark was present in carbohydrates. As an explanation the authors discuss the reversibility of reactions connected with respiration. Recently Thimann (90) has suggested that a reversion of some fermentation reactions might serve for the initial fixation of free carbon dioxide in photosynthesis. It is, therefore, of interest to note that no radioactive carbon was found in the keto acids, such as pyruvic acid. The dark reaction detected is apparently of enzymatic nature. Hydrogen cyanide inhibits the reaction and the possibility of an ordinary chemical interchange has been excluded experimentally.

#### ABSORPTION OF CARBON DIOXIDE BY THE PLANT

According to Willstätter & Stoll (1918) leaves of several plants can reversibly bind large quantities of carbon dioxide independently of the chlorophyll content. To settle the question whether this surplus of "fixed"  $\text{CO}_2$  is connected with the assimilatory apparatus, Smith (81) has analyzed the substances responsible for the absorption and has found them to be inorganic magnesium and calcium salts with alkaline reaction. As there exist numerous plants in which carbon dioxide is not stored in this manner (31, 32) we have to assume that such carbon dioxide absorption by plant material is more or less accidental and not an essential feature of the photosynthetic mechanism. If the substance which, in the dark, binds carbon dioxide is present in amounts equal to or larger than the amount of chlorophyll, the measurable production of carbon dioxide by respiration should be diminished for a moment after a period of intense illumination. We should expect to see a "refilling of the reservoir." In McAlister's (53) experiments, where  $\text{CO}_2$  exchange at rather low partial pressures of this gas has been accurately recorded, the effect, if existent at all, is very small.

Under physiological conditions pure chlorophyll (a + b) has no affinity for carbon dioxide (73, 81). The view, taken by the writer in 1933, that chlorophyll is attached to the reducing mechanism only as a sensitizer appears now to be accepted by the majority of investigators (2, 3, 17, 23, 49, 65, 96, 98).

The question arises whether in green plants the substance which

binds carbon dioxide with such avidity (13) is present only in the light. This possibility is not yet excluded, though we know that carbon dioxide is reduced by some organisms in the dark. (See above.)

#### THE PLASTID GRANUM AND THE PIGMENTS

On the structure of the chloroplast two recent reviews have appeared (22, 94). It is now certain that "the chloroplast consists of a colorless stroma or ground substance in which small discs (grana) of chlorophyll-impregnated cytoplasm are embedded." The chromatoplasma of the blue-green algae, which is morphologically different from the plastids of green plants, has also this granular structure (30). Contents and structure of the grana are the subject of papers from the botanical laboratory at Leiden (3, 36, 58, 64).

A layer one or two grana thick will absorb half of the incident red light. After extracting the pigments and lipoids the residue of the granular substance was weighed and estimated as protein. Calculation showed that for each chlorophyll molecule one protein unit with a particle weight of 17,000 was present (58). Other and probably less accurate figures were found by Granick (34, 35). Granick estimated that there are approximately thirty chlorophyll molecules for each protein molecule of the chloroplast, if the protein is assumed to have a molecular weight of 100,000. Compared with the data of the Dutch investigators this would imply a five-fold higher pigment concentration.

Weier's (95) interesting studies on the reduction of silver nitrate in the chloroplasts, which occurs when leaves are killed in boiling water, "indicate the normal presence in the cell of strongly reacting substances, which, though very close together, are separated in some manner so that if they do react, it is at a very slow and controlled rate."

The author concludes that "ascorbic acid is the substance responsible for the reaction observed in the chloroplast." The experiences of Weier (95) and of Strain (89)—oxidation of carotinoids—indicate the presence of a semipermeable membrane around the granum, an assumption consistent with many other facts discussed in the following sections.

Aqueous solutions of the pigments united to protein have been prepared from green cells (82) and from purple bacteria (22). The absorption spectrum of such (photosynthetically inactive) solutions agree with those of the leaves or of the bacteria.

A comparison between the absorption spectra of the living cells and photosynthesis in light of different wave lengths has proved again that only light absorbed by the chlorophylls is used in photosynthesis (9, 20). This fact is also convincingly demonstrated when green algae or purple bacteria are allowed to grow in a spectrum, a method first introduced by Dangeard. The criticism (1a) of those papers (56, 57) in which the photosynthetic effect of light absorbed by carotenoids, especially xanthophyll, is advocated, still holds good. The technique used in the more recent studies has not been sufficiently improved to justify the contentions made. The objections to the xanthophyll hypothesis are supported by Seybold's (80) observation that green algae contain the same amount of green and yellow pigments as brown and red algae when compared on a dry weight basis. Only the distribution of the pigments is different.

Further information relating to the chloroplast and the chloroplast pigments is contained in the following references:

Studies on the formation of pigments in etiolated seedlings (4, 79, 88, 89); pigment formation in light of different intensity and color (37, 38, 79, 88); review of the chemistry of chlorophyll (84); chemistry of bacteriochlorophyll (15, 16); quantitative estimation of bacteriochlorophyll (63); reversible oxidation and bleaching of chlorophyll (71, 72); light absorption and constitution of chlorophyll derivatives (86); leaf xanthophylls (88); spectrum of the chloroplasts (1); fluorescence spectra of photosynthesizing cells (91); the part played by carotenoids in sexual differentiation of algae (54), and in phototaxis (8).

#### THE DARK REACTIONS IN PHOTOSYNTHESIS

Investigations of the influence of heavy water (99 per cent) on assimilation have been continued with important results (10, 68, 70). In continuous light the rate of photosynthesis in *Chlorella vulgaris*, measured by oxygen production, is retarded 60 per cent. If, however, the dark reactions are given time to run to completion by using intermittent (flashing) light the rate of photosynthesis (the amount of oxygen produced per flash) is equal in ordinary and heavy water. This indicates that water enters into one of the dark reactions of photosynthesis. There is little doubt that the dark reactions are catalyzed by specific enzymes, and it is known that the rates of enzymatic reactions are influenced by heavy water.

The fact that the photosynthetic activity of older cells is considerably less than that of young ones has been observed several times [e.g., (14) etc.].

Pirson (67) reported that the cells from nine-day-old *Chlorella*

cultures were 50 per cent less effective in high light intensity than those from four-day-old cultures. Van Hille (40) investigated the factor responsible for this behavior. He found that the assimilatory activity of old cells could be partly restored by addition of nitrate which apparently had been exhausted in the old cultures. Independently Pirson found that cultures grown in a medium with very low nitrate content yielded cells with low assimilatory power. Photosynthesis was increased enormously by the addition of nitrate, with only an insignificant formation of new chlorophyll. Normally the effect of nitrate in old cells is made manifest by a slowly increasing rate of photosynthesis, reaching its maximum only after ten to twelve hours. But Pirson also found almost instantaneous acceleration, due in all probability to direct nitrate reduction. The writer has confirmed these observations. The synthesis of additional enzyme participating in the dark reactions is a plausible explanation for the activation which reveals itself only after many hours (40). That such a synthesis occurs in the dark independently of any changes in chlorophyll content or chlorophyll activity at low light intensities follows not only from the old experiments of Willstätter & Stoll with autumnal leaves but also from recent ones (23) in which part of the enzyme system was artificially destroyed by the specific action of very dilute hydrogen peroxide which does not harm the activity of the chlorophyll. In both cases recovery took place within twenty-four hours.

Pirson (67) discovered a remarkable specific effect of potassium and manganese salts upon photosynthesis of *Chlorella* grown in media with very low concentrations of these salts. The photosynthetic rate is increased 50 to 100 per cent during the first thirty minutes after addition of the deficient salts. Potassium ion inhibits the (accelerated) respiration and retards the effect of manganese in manganese-deficient cells; its mode of action is very probably connected with an effect on permeability. Manganese may be necessary for some catalysis as is, for example, the case in fermentation. For important details, the reader is referred to the original paper.✕

The question as to whether or not the dark reactions in photosynthesis involve a peroxide decomposition has been settled at least partially. It has been shown that the intermediate compound yielding molecular oxygen is not *hydrogen peroxide*, which substance has been hypothetically included in practically all of the mechanisms of carbon dioxide reduction proposed between the years 1926 and 1936. If we

recognize that photosynthesis is possible with four quanta of red light, hydrogen peroxide is excluded for thermodynamical reasons [Wohl (98-102)]. In addition, the capacity of algae to decompose hydrogen peroxide has been found to vary quite independently of changes in the photosynthetic activity of the cells [Emerson & Green (12), van Hille (40)]. The most conclusive evidence is furnished by studies on the effect of cyanide on photosynthesis, respiration and hydrogen peroxide decomposition by *Scenedesmus Spec. D3*.

In these organisms photosynthesis is only slightly inhibited by cyanide concentrations which cause a practically complete inactivation of catalase. In the presence of cyanide the enzyme responsible for oxygen production in photosynthesis can be selectively destroyed by 0.0001 *M* hydrogen peroxide (23). Whereas hydrogen peroxide is excluded as an intermediate product in photosynthesis, the probability has increased that a different peroxide is formed as an intermediate product and that this is split with the help of a catalyst which differs from ordinary catalase perhaps only in the protein part determining its specificity.

The arguments in favor of this assumption are here summarized: (a) Photosynthesis (29) as well as catalase activity (47) is inhibited by azides. Both catalase and the enzyme functioning in the Blackman reaction are specifically poisoned by certain compounds, one of which is sodium azide. (b) Some years ago Gaffron (24) found that carbon monoxide inhibits the action of the photosynthetic apparatus as long as the cell remains in a reduced state, and a year later Keilin & Hartree (47) demonstrated that it forms a compound with catalase, when the catalase is in the reduced state (48). (c) Another argument is the specific sensitivity of the Blackman reaction to 0.0001 *M* hydrogen peroxide. (d) Finally, the assumption that ordinary catalase and the "Blackman enzyme" are related is in agreement with the results of modern enzyme chemistry that similar catalysts may have identical prosthetic groups.

*The relation between photosynthesis and respiration.*—This has always been a matter not only of methodological but also of theoretical importance. The questions whether at low light intensities the so-called "compensation" will alter the nature of carbon dioxide reduction and whether at high light intensities respiration may be directly increased to a measurable and significant extent have again been discussed by McAlister (53) and by Gaffron (23), because they could find no direct influence whatever of photosynthesis on



normal respiration. Gaffron therefore concluded that, in case a light-induced respiration [*e.g.*, in the sense of Franck & Wood (1a)] existed at all, it should be different from, and not connected with, the ordinary respiration. By later experiments the occurrence of different oxidation reactions taking place inside the assimilatory apparatus has been demonstrated (29). Under certain circumstances the photoperoxides will react immediately with hydrogen donors, the production of oxygen then being partially or completely inhibited.

*The reduction of carbon dioxide with molecular hydrogen in green plants* (28, 29).—This reduction is the most clear-cut example of just such a total "inhibition" of oxygen production. *Scenedesmus* cells kept for some hours in an atmosphere of hydrogen and subsequently illuminated will behave differently from control cells incubated in nitrogen. They do not produce oxygen but absorb hydrogen instead, about two to three molecules of hydrogen for each molecule of carbon dioxide absorbed. The reaction depends on the light intensity in a peculiar way. Too high a light intensity apparently causes a reoxidation of the assimilatory apparatus which then starts to produce oxygen, hydrogen simultaneously becoming again an inert gas for the plant. Luminous bacteria, the most sensitive and most specific reagent for oxygen, failed to indicate even the intermediate production of oxygen during the reduction of carbon dioxide in the "reduced" plant cell. Thus photosynthesis of green plants has been brought into line with the same process in purple bacteria, confirming van Niel's early assumption that both processes might turn out to be identical in principle.

*Studies of the induction period in photosynthesis*.—These have lately been the most successful means of analyzing the sequence of reactions involved in carbon dioxide assimilation. The objections of Emerson (1a, p. 539) have been refuted, and the earlier observations of Gaffron (24) have been confirmed and extended. It is now certain that induction periods under aerobic and anaerobic conditions have different causes. After a longer period of anaerobiosis the assimilatory apparatus is more or less charged with reducing substances. If it is not already damaged by secondary reactions, full photosynthesis commences immediately on subsequent illumination. However, on account of the abundance of hydrogen donors the oxidation reactions mentioned above will occur, permitting only part of the oxidized products of photosynthesis to escape as molecular oxygen. In this

event the *assimilatory quotient*,  $-\text{CO}_2/+\text{O}_2$ , believed to be always unity, loses its meaning completely, because the photooxidations may even take a course in which production of carbon dioxide exceeds its reduction (29). On the other hand, McAlister's measurements on induction periods (53) support the assumption that the normal aerobic induction period is caused mainly by a reversible oxidation in the dark of a catalyst (26) which must first be reduced in the light in sufficient amount to split all peroxides formed at the respective light intensities. These measurements show a convincing proportionality between the amount of light lost for photosynthesis during the induction period and the incident light intensity.

Most promising for further analysis of induction periods are the methods and the results of Blinks & Skow (7). They followed the time course of photosynthesis from second to second after a sudden change from light to darkness or *vice versa*. Their results agree with the observations and explanations mentioned above and especially confirm the fact that after longer periods of anaërobiosis, oxygen will not be released from the plant cell but will temporarily be used for oxidation reactions. In addition, they have detected a kind of "explosive" oxygen formation which momentarily precedes the effect of anaërobiosis just mentioned. As these authors suggest, this may be due to the direct effect of light-energy upon an oxygen-releasing substance. [Compare Kautsky (45, 46) and Ornstein, *et al.* (65).]

*Studies on the metabolism of the purple bacteria.*—Nakamura (59) measured the influence of light upon the oxygen uptake of suspensions of *Rhodobacillus palustris* in the presence and absence of organic substances. It had already been ascertained that light may inhibit the absorption of oxygen by *Athiorhodaceae*. In *Rhodobacillus* the decrease of respiration by the light is very pronounced, 50 to 100 per cent, if the washed bacteria are forced to respire their own metabolites. Light has no effect, however, on the respiration of such substances as fatty acids, which are known to be the preferred substrates for carbon dioxide assimilation by purple bacteria in the absence of oxygen. Nakamura assumes that the diminution of oxygen uptake in the light is the result of "oxygen production." So far there is no evidence that molecular oxygen is produced by any of those bacteria in which bacteriochlorophyll is the light-absorbing substance. Formation of molecular oxygen is improbable for thermodynamical reasons (*a fortiori*, therefore, the intermediate formation of hydrogen peroxide).

All known facts point to the opposite result, particularly the discovery that green algae under anaërobic conditions can utilize hydrogen. The diminution of the oxygen uptake in the light in Nakamura's experiments rather demonstrates that photoperoxides enter into competition with molecular oxygen for the available hydrogen donors (26). The writer is inclined to agree with Nakamura and Shibata only to the extent that the difference between photosynthesis in plants and in purple bacteria lies not in the actual reduction of the carbon dioxide molecule, but in the disposal of the oxygen.

The experiments of Roelofsen and Gaffron on the utilization of hydrogen by purple bacteria<sup>2</sup> for the reduction of carbon dioxide, organic compounds, nitrate and oxygen have been extended by Nakamura (60) to such substrates as nitrites, fumaric acid, methylene blue, sulfur, and thiosulfate. The latter reactions are of interest in view of the controversy between van Niel and Gaffron on the theoretical significance of hydrogen sulfide production in purple bacteria.<sup>3</sup>

Oxyhydrogen reaction in green plants (29); in bacteria (97, 105); effect of hydrogen sulfide on photosynthesis in *Oscillatoria* (61); assimilation in plants adapted to low or high intensities (31, 32, 79, 85).

#### QUANTUM EFFICIENCY. LIGHT REACTIONS

It is obvious that among measurements of quantum efficiency only those of the highest efficiency recorded, *i.e.*, with the lowest number of quanta per molecule of carbon dioxide reduced, are of importance. Since 1936 the high quantum efficiency observed in Warburg & Negelein's famous experiment with *Chlorella* has been duplicated by Emerson (private communication), Franck (private communication), Ornstein, *et al.*, (65) who have reported from four to six quanta for each molecule of oxygen produced. The writer agrees with Eymers & Wassink's (14) opinion, that it does not matter much whether the oxygen production is linked with reduction of carbon dioxide or with a reduction of "intermediates" formed by respiration. These experiments have been made with the manometric method. Using an entirely different method Arnold (2) has established a conversion of radiant into chemical energy in photosynthesizing cells with an efficiency of at least 45 per cent. This is

<sup>2</sup> *Ann. Rev. Biochem.*, 5, 541 (1936).

<sup>3</sup> *Ann. Rev. Biochem.*, 6, 606 (1937).

equivalent to the utilization of six quanta for the reduction of one carbon dioxide molecule. He measured the difference in the heat produced by photosynthesizing and by inactivated *Chlorella* cells when illuminated with monochromatic red light. Manning, *et al.* (50, 52), using *Chlorella pyrenoidosa*, reported only one fifth or less of the efficiency measured by Warburg and Negelein. This low efficiency may be only partly due to the rather inadequate method employed. (The plants were illuminated for an entire hour; carbon dioxide and oxygen changes were measured by gas analysis; the corresponding volumes of oxygen and carbon dioxide obtained differ widely.) The low efficiency can probably also be ascribed to conditions within the plant. We know now that the photosynthetic oxygen may disappear by way of side reactions and, also, that different strains, belonging to the same species, may react quite differently. But at the time when these experiments were made the existence of a variable light-respiration was unknown.

Another case of an unsatisfactory low quantum efficiency is found in a paper by Eymers & Wassink (14) on the photochemical carbon dioxide assimilation in purple sulfur bacteria. These authors took every possible care to work with bacteria in the most active state, and to use suspension media and substrates likely to give the most consistent results. Yet they found quantum numbers between ten and thirty, the average of "good" measurements being about fifteen quanta for each carbon dioxide molecule absorbed. Such results with purple sulfur bacteria could have been predicted, had the authors considered the established facts concerning the dissimilation reactions in *Thiorhodaceae* which are known to be complex and accelerated by light. Only when these dark processes have been quantitatively analyzed can the determination of the total balance of carbon dioxide exchange prove useful for quantum efficiency estimations in *Thiorhodaceae*.

Because the sulfur-free, purple bacteria have practically no measurable dark metabolism if suspensions of washed cells are used, determinations of the quantum efficiency for the reaction  $\text{CO}_2 + \text{H}_2 + \text{light} \rightarrow (\text{CH}_2\text{O}) + \text{H}_2\text{O}$  appear less ambiguous. Studying this reaction French (19) arrived at an average value of seven quanta per molecule of carbon dioxide reduced, the lower limit being in the neighborhood of 4.4 quanta. When organic substances were present, stored in the bacteria or in the medium, the number rose to fifteen quanta, indicating a counteracting dark fermentation or

the occurrence of photosynthesis in which the actual carbon dioxide turnover cannot yet be determined.

To the writer it appears that the reduction of carbon dioxide with hydrogen in purple bacteria should require a minimum of four quanta, in spite of the fact that thermodynamically the process may occur without light, since the same reaction can take place in green algae, as mentioned in the preceding section. In addition we have the observations of Nakamura (59), who claims that for every four to five quanta absorbed by an illuminated suspension of respiring purple bacteria the uptake of one molecule of oxygen is prevented. As yet nobody knows what reactions are involved in the effect studied. In French's experiments a previous shaking of the bacteria in air caused the bacteria to give the highest efficiency. For that reason Nakamura's figures should be remembered in spite of the objections to which his experiments are open. [See also the discussion in Eymer & Wassink's (14) paper.]

The hope of tracing the course of light reactions in photosynthesis by measuring fluorescence (45, 46) has to be abandoned. To the rather long list of critical discussions in this connection (1a) may now be added that of Ornstein *et al.* (65, 92). For the first time they have compared fluorescence with photosynthesis in the same sample of cells. Only 0.15 per cent of the absorbed light is emitted as fluorescence by the chlorophyll of the living plant and 0.005 per cent by the bacteriochlorophyll in purple bacteria. The only parallelism observed between photosynthesis and fluorescence is a sensitivity to narcotics. If the rate of photosynthesis is reduced by urethane the intensity of the fluorescence increases, an observation which confirms Gaffron & Wohl's (1a) assumption that the effect of narcotics consists in a lessening of the energy transfer from the activated pigment to the assimilatory system. The 0.15 per cent of all activated chlorophyll molecules which normally show fluorescence has to be considered as belonging to a fraction of "insulated," normally inactive molecules. These "lost" molecules may react in some way with oxygen, as Kautsky and Franck & Wood (1a) have demonstrated, but this has nothing to do with carbon dioxide reduction. With the exception just mentioned the Dutch investigators have found strict proportionality between incident intensity and fluorescence, no matter at what intensity photosynthetic saturation in the illuminated cells was reached, or how strongly photosynthesis was inhibited by cyanide.

## THE PRODUCTS OF PHOTOSYNTHESIS

Even yet nothing is known regarding the organic compound first formed in photosynthesis. The firm belief that it should be a substance of the formula  $(\text{CH}_2\text{O})_x$  is still supported by the facts showing the balance of gas exchange in the stationary state to be either one oxygen or two hydrogen molecules for each carbon dioxide molecule reduced. The interesting possibility of building an entire glucose molecule in one uninterrupted process with the aid of an underlying protein pattern has been discussed by Wohl (100). Such a suggestion would not only account for the failure to find any formaldehyde in assimilating cells but also might serve to explain the abnormal temperature coefficients observed in photosynthesis under certain conditions. Furthermore, it would require less energy than the formation of free formaldehyde.

An entirely new experimental approach to the problem has been opened by the production of radioactive carbon in the Berkeley cyclotron. Boron is bombarded with 8 MEV deuterons and is transformed into  $\text{C}^{11}$  which has a half-life of 21.5 minutes.

This radioactive carbon dioxide, mixed with ordinary carbon dioxide, has been used in assimilation experiments of short duration. The entire plant material was then chemically separated into fractions which were examined for the presence of radioactive carbon. In this way Ruben, Hassid & Kamen (75) were able to show the fractions into which the newly assimilated carbon dioxide had gone. It is very surprising that in these experiments in which the plants had been exposed to the "labeled" carbon dioxide for periods of continuous illumination lasting fifteen to seventy minutes, only 20 per cent of the total carbon dioxide fixed was later found in the carbohydrate fraction. The other 80 per cent was present in a fraction which was neither of carbohydrate nor of keto acid composition. By extracting the chlorophylls and transforming them into phytorhodins, etc., these authors found that about 0.04 to 0.08 per cent of the carbon assimilated had been built into the pigment molecules. This would indicate a slight synthesis of chlorophyll during the time of the experiment.

## THEORETICAL CONSIDERATIONS

Any theory of photosynthesis must account for the following well established observations which are, after all, the most difficult to explain: (1) Reduction of carbon dioxide with only four quanta

of energy, *i.e.*, with practically no loss of energy. (2) The excellent yield of this reaction under conditions where on the average each chlorophyll molecule absorbs considerably less than four quanta during the experimental period, and this in spite of the absence of any long induction time necessary to establish an equal distribution of intermediate products. (3) The rapid light saturation in flashing light, *i.e.*, under circumstances where the known dark reactions are not the limiting factor.

All the various hypotheses proposed before 1936 have constantly disregarded these facts. The critical reviews of Emerson (1*a*) and of Gaffron & Wohl (1*a*), in which this has been emphasized, have brought about a change. Today there is agreement that intermediate products of too high an energy content, for instance hydrogen peroxide, can not play a part in photosynthesis. As an explanation for the second and third points a collection of quanta in a complex consisting of a great number of chlorophyll molecules and a transfer of this energy to the actual reducing mechanism has been proposed, the "effective optical assimilatory unit," for discussion see (1*a*). As an alternative explanation for the third point Franck & Wood have suggested a photooxidation opposing assimilation [see (1*a*)]. In consequence of these discussions Franck & Herzfeld (17) have published a new theoretical paper. With the help of some special assumptions (*e.g.*, mobility of the chlorophyll molecule upon the surface of proteins, chain reactions initiated by photolytic decomposition of percompounds, etc.) and an elaborate mathematical calculation they proceeded to depict the process of photosynthesis.

Wohl (99) devoted one out of a series of five papers on the theory of photosynthesis to an extensive criticism of Franck & Herzfeld's theory, leaving no doubt that among other shortcomings it did not comply with the second point mentioned above. The work of Wohl (98, 99, 100, 101, 102) appears to be the most careful theoretical survey of the available data hitherto presented. It contains information about energy relations, many experimental suggestions, a discussion of the abnormal temperature coefficient of photosynthesis at low temperatures, a special model of a twenty-four-quanta process for a direct formation of glucose with suggestions as to how this hypothesis may be tested experimentally. His most interesting contribution, however, is a calculation made for the purpose of demonstrating that a special type of a chemical-photosynthetic unit is possible. This hypothesis implies the existence of a large

amount of sensitizable molecules which act as carriers of energy from the pigment to the reducing agent. Ornstein *et al.* (65) believe that such a mechanism has to be adopted. They have developed mathematical equations, corresponding to a mechanism similar to Wohl's, which agree with their experimental results.

An assimilatory unit, which collects quanta by means of carrier molecules, is of great interest, since an optical unit has become less probable for the following reasons. Recently a model of the hypothetical optical unit has been discovered in which quanta migrate simply as electronic waves. Scheibe (77, 78) has found that certain fluorescent dyes associate in concentrated solution in such a manner that the energy absorbed by one molecule is easily transferred to other quite distant molecules and is then re-emitted as fluorescence. These experiments might be taken as a most welcome proof for the reality of the alleged optical unit in the plant were it not for the fact that the unassociated dyes display a conspicuous change in their absorption spectra as they become associated. A new characteristic absorption band becomes visible. This is contrary to the behavior of chlorophyll for no such new band appears in its spectrum when it is concentrated, for example, as it is in the granum.

Franck and Teller (18) believe that this difference is evidence against the existence of an optical unit. Further studies on the state of the chlorophyll in the granum (3, 36, 58) are therefore highly important and they should be compared with the experimental and theoretical results on energy transfer in large molecules (25, 44, 55, 93, 96). Quite recently, modifying his former specific theories, Franck has assumed that each step in the photosynthetic process is reversible (personal communication). If, in the dark, a series of oxidation reactions proceeds through the same steps, but in opposite direction, as the light energy drives the reduction of  $\text{CO}_2$ , then an equal distribution of all the intermediate products might be maintained inside the assimilatory mechanism. Assuming further that the very slow back-reactions increase in speed with the concentration of the products of illumination, the second and third points can be explained without a "unit." Under such circumstances the ratio of chlorophyll to carbon dioxide bound could again be unity.

To explain induction periods, Gaffron (26) had postulated reversible oxidations and reductions of catalysts involved in assimilation. In the meantime this has been demonstrated experimentally (28, 29). These experiments, particularly the proof of a special



light-respiration, lend strong support to Franck's newest assumptions. But the phenomena of solarization; of light saturation; of light respiration as displayed by the difference in the effect of glucose on photosynthesis under aerobic and anaerobic conditions; of adaptation of the cell to low and high light intensities; of the effect of potassium salts, and of the sensitivity to mechanical injury can easily and simultaneously be explained by the assumption of a semi-permeable membrane encompassing the assimilatory mechanism or perhaps the granum. A burning of organic substances trapped inside the assimilatory chamber does not necessarily require a strict reversibility of the photosynthetic mechanism.

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## MINERAL NUTRITION OF PLANTS

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The subject of "Mineral nutrition of plants" covers such a broad field of investigation that the mere citation of all the papers appearing in the past few years dealing with the problems involved would alone probably require more space than has been allotted for this chapter. It becomes necessary therefore to impose a somewhat arbitrary restriction upon the number of papers selected for brief review. The purpose is to deal primarily with such material as will indicate the general trends of progress in the field and to present quite briefly some results of the investigations in those phases of the subject which appear to command the greatest interest.

### ABSORPTION AND ACCUMULATION OF SALTS BY CELLS

The investigations directly concerned with the processes of salt absorption by plant roots and the nature of the mechanisms by which this is accomplished constitute a most important phase of the whole field of mineral nutrition of plants. This is indicated by the keen interest manifested in this field by plant scientists in general and by plant physiologists and soil scientists in particular. That much important progress has been made along these lines in the past decade is indicated by the long series of important researches dealing with these problems reported in previous reviews (24, 25, 26, 38, 71).

While it is not within the scope of this review to dwell upon the work previously reported, mention should perhaps be made of a few of the outstanding conclusions as a background for what is to follow relative to the problem of ion absorption and salt accumulation. Strong emphasis is given to the conclusion that the quantitative accumulation of salts is determined by a state of intense metabolic activity by the accumulating cells, an activity which is intimately associated with aërobic respiration and indicated by the production of carbon dioxide, although no stoichiometric relationship has been found between the salts accumulated by the assimilating cells and the yield of carbon dioxide. Nor does mere carbon dioxide production by roots explain salt accumulation but involves rather metabolic activities which are associated with active aërobic respiration. It is generally

accepted, and the experimental evidence is quite clear, that salt accumulation in actively assimilating cells proceeds against concentration gradients and cannot be adequately accounted for by the principles involved in the Donnan equilibrium or by exchange mechanisms, particularly when cation and anion accumulation proceeds simultaneously. Although in this connection Petrie (52) in his recent investigation of the intake of ions by carrot tissue in relation to hydrogen-ion concentrations, suggests that while the Donnan system may be operative in determining the amounts of ions absorbed by the tissue, other factors play a very important part. It is emphasized that the absorption and accumulation processes involve the expenditure of energy in work accomplished. While the energy necessary for these processes is readily accounted for through external sources or its release by the oxidation-reduction processes in the actively metabolizing cells, the mechanism by which such energy is applied in salt accumulation is quite obscure.

Hoagland & Broyer (27) call attention to the importance of eliminating complications which may arise through root and shoot relationships in considering many of the fundamental questions of salt absorption by plant roots. In their comprehensive studies of the problems of salt accumulation with particular reference to the metabolic processes they make use of excised root systems of barley plants and give special consideration to the methods employed. The importance of an adequate realization of the nutritional status of the tissues is strongly emphasized. An excised root system cannot manifest maximum potentialities for salt accumulation when the metabolically active cells do not predominate in the system. Loss of salts from inactive cells may obscure accumulation by active ones and misleading conclusions may be reached relative to the factors which affect the process.

Active aerobic respiration of roots reflects energy exchanges which are essential to the accumulation of both cations and anions by the cells. Thus experimental evidence (27) leads to the conclusion that an adequate oxygen supply is essential to maintain the cell activities associated with salt accumulation over an extended period or to retain the salt already accumulated. Collander & Holmström (16) also point out that active salt absorption by metabolically vigorous cells is strongly inhibited by a deficiency of oxygen. On the other hand, it is strongly emphasized that active absorption of salts is essential to maintain the physiological efficiency of cells which are functioning at high metabolic rates. The experiments of Hoagland & Broyer (27) illus-

trate in a striking manner the importance of the relation between metabolic cell activity and salt accumulation by excised root systems, and the authors are led to the conclusion that there is no fundamental variance in the nature of salt accumulation in these excised root tissues and in storage tissues. They find justification also for strongly emphasizing the point that accumulation of salts by the living cell involves much more than merely a question of cell permeability. Beckenbach, Robbins & Shive (6) have also pointed out with justification that, within the limits of ionic concentrations employed in the experimental setup, the rates of penetration of ions into the plant roots are not significantly influenced by the nature of the membranes of the absorbing cells.

While a high metabolic rate maintained by the energy derived from aërobic processes clearly is an essential feature of actively accumulating cells, this alone may not be effective when cells have become fully mature, according to Prevot & Steward (57). Growth trends and development which intervene between the nonvacuolated meristematic cell on the one hand and the active, highly vacuolated, fully developed cell, on the other, appear to be associated with a graded intensity of ion accumulation. These authors produce quantitative evidence which leads directly to the conclusion that the degree of intensity or the graded activity in ion accumulation by the tissues of the absorbing root is determined by the stage of development which these tissues have attained. Thus a pronounced, longitudinal gradient of accumulation along the axis of the root is apparent, with highest activity and maximum concentrations shown for samples nearest the apex of the root and with a progressive and consistently decreasing gradient of concentration toward the oldest tissues examined. This gradation is the inevitable consequence of the progressive development of cells from the apex of the root, the whole surface of which extending from the apex to the point of emergence of secondary roots represents a potential ion absorbing surface. The metabolic basis for such a gradient of salt accumulation is furnished by the fact that the tissues in the regions of most active accumulation exhibit also the highest oxygen requirement. The outstanding fact is that the cells which are capable of most active growth, and those which show the highest capacity for vacuolation and extension show also the highest capacity for the absorption of ions of both signs. The results of anatomical study are in accord with the view that the relative efficiency of the tissues nearest the root apex must be ascribed to the preponderance

of metabolically active cells endowed with a high capacity for dividing and rapid enlargement.

Steward (72) recognizes that not all cases of salt or ion absorption by cells are of the same kind and refers specifically to two types of ion uptake. In the case of alkali halides, commonly both anions and cations are absorbed simultaneously, and frequently in approximately chemically equivalent quantities. Here loss of other ions, except carbon dioxide, does not occur and the result is a net gain of total salt content which entails simultaneous movement of anions and cations not in accord with concentration gradients. This type of absorption has been designated "Primary Absorption." It represents a process tending to increase the total energy of the cell, therefore involves work accomplished and is essentially a property of the living system. It is accompanied by vigorous metabolic activity and is clearly dependent upon a capacity for growth. Another type of ion uptake designated "Induced Absorption" occurs in cells which manifest subsided growth and exhibit a correspondingly lowered degree of metabolic activity but may still absorb ions through exchange mechanisms by replacement of those previously accumulated. The importance of distinguishing between the different types of absorption as outlined is strongly emphasized but attention is also called to the fact that the relation between growth and ion absorption and salt accumulation cannot, as yet, be attributed to any given metabolic reaction.

In this connection attention should be directed to the important work of Jenny & Overstreet (34, 35) dealing with contact exchange mechanisms in the absorption and accumulation of ions by the plant roots, especially since they regard the soil solution as identified with the nutrient solution of the plant physiologist. The hypothesis here proposed, and supported by experimental evidence differs from the soil solution theory in that it is based on the phenomenon of ion interchange which may take place between two surfaces which are in contact as, for example, the root absorbing surface in contact with the particles of a colloidal clay suspension carrying adsorbed cations. Since ions adsorbed on a colloidal surface remain and oscillate within the field of force emanating from the colloid it is conceived that two separate colloidal particles may approach so closely that the oscillation spaces interpenetrate. This represents the condition, for contact exchange of ions between two different particles, under which the reaction as such is independent of the nature of the intermicellar solution. Since contact exchange involves the mutual transfer of ions, a distinc-

tion is made between contact intake by the roots immersed in cation clay suspensions and contact depletion, which involves contact exchange of the nutrient cations (previously accumulated) of the root for the hydrogen ions of the clay suspension. Quantitative evidence is presented to demonstrate the existence of such contact phenomena between plant roots and colloidal clay particles, and the evidence suggests that present concepts of the mechanism of mineral absorption by plant roots must be modified and extended. Results point to the conclusion that the absorption of nutrient ions is not a unidirectional process but that movement of ions of the same species into and out of the root may take place simultaneously, so that "accumulation and depletion represent only net effects of ionic movements."

Although it is well established that ions will accumulate in plant cells against an apparently steep concentration gradient, the absorption of any one ion from a mixture of ions in a nutrient substrate and the accumulation of the corresponding element within the cells of the plant are nevertheless functions of the concentration of the ion in the substrate (6, 14). This is directly supported and emphasized by the work of McCalla & Woodford (42) who find that under conditions of growth where the supply of an element is limited, the absorption of the element by the plant is dependent upon the external concentration of the element. However, where the concentration of an element in the substrate is sufficient to provide maximum absorption, increase in absorption as a result of increased concentration in the substrate would not be expected. They also find that limiting the supply of one nutrient ion has the effect of increasing the absorption of another ion of the same sign, or of decreasing the total intake of ions of the opposite sign. While there is a marked tendency toward a maintained balance between total anions and cations, the balance always appears to be in favor of the anions. The results as reported reflect the interrelations between the various nutrient elements regardless of the exact mechanisms of absorption or of the external and internal factors affecting this process.

It is indicated by the work of Beall (4) that the greater the degree of ionization of acids (six acids tested as constituents of a nutrient substrate) the more rapid is the net absorption. Net absorption from organic acid solutions is generally less than from corresponding concentrations of mineral acids, and this is in general agreement with the order of dissociation of these acids. Furthermore, the tissue content of any element may be directly or inversely related to the concentration



of other elements in the substrate, depending upon certain of their known chemical properties and the nature and degree of their physiological effectiveness.

Brooks (10) readily admits that, as yet, there is no adequate solution to the problem of why ions accumulate in cells in higher concentrations than in the surrounding solution, and enlarges upon a previously proposed mechanism of selective penetration of ions based upon ion mobility and exchange in a membrane described as "mosaic" in structure, under conditions of a nonequilibrium state. A cell while metabolically active and growing cannot attain a state of equilibrium. Brooks suggests that since protein molecules have high valency it is possible that large numbers of both basic and acidic groups could be displaced from a single molecule by cations and anions of nutrient salts, which would result in the accumulation of these ions. The mosaic idea is based upon this theory rather than upon the possibility of definite delimited areas of cation or anion permeability. Since proteins are amply supplied with basic and acidic groups these could be displaced simultaneously by nutrient cations and anions. The base-protein and the acid-protein thus formed could then combine with basic and acidic groups of adjacent molecules and thus migrate through the protoplasm to the sap or vacuole and there exchange with hydrogen ions and bicarbonate ions which are always present while the plant is alive. Evidence is cited to show that penetrating substances do form temporary associations with the protoplasm before passing into the sap. The sap of the cell thus plays a minor role.

In this connection Mazia (49, 50) presents evidence that cations such as calcium, strontium and barium can be more or less firmly "bound" by *Elodea* protoplasm, presumably to organic constituents, but that they differ in their tendencies to be bound and are capable of replacing each other by exchange mechanism. While further evidence is produced which indicates that calcium may replace sodium or potassium from combination with an organic anion, it gives no indication of the nature of the compounds to which calcium is bound within the cell. It only indicates the existence of such compounds and their probable role in ionic exchange and accumulation against diffusion gradients.

Brooks (11, 12) carried out exploratory experiments on the penetration of radioactive potassium, sodium, rubidium and bromine, which "reveal hitherto unknown steps of the process of penetration of these ions into living cells." Brooks (10) has also shown the importance of

separating the protoplasm from the sap and of studying these portions separately, in any endeavor to gain an insight into the mechanism of penetration and accumulation. He finds that radioactive potassium (12) first accumulates in high concentrations in the protoplasm of *Nitella* cells and later diffuses into the sap, and emphasizes the point that concentration changes in the large vacuole represent the last link in the chain of ion movement.

Further study of the problem of electrolyte accumulation has been made by Jacques (30) and by Jacques & Osterhout (33) with special reference to the replacement of ammonia by sodium and potassium in *Valonia macrophysa* in sea water, and to the accumulation of iodine by *Halicystis Osterhoutii* and *Valonia*. In general the results of these investigations harmonize with the views previously presented, that the movement of a base such as potassium through the protoplasm is determined by the difference of the activity products  $(K)_o(OH)_o$  and  $(K)_i(OH)_i$  where  $o$  and  $i$  represent sea water and cell sap, respectively. An elaborate mathematical consideration is presented which serves to show how the relative rates of entrance of sodium and potassium into the cell are determined.

In considering the various theories dealing with the intake and accumulation of ions the recent reviews by Pirschle (55, 56) on mineral metabolism, particularly those sections relating to the absorption of electrolytes, are quite helpful. He discusses, at some length, the theory of Lundegårdh (39) and compares it with Osterhout's (31, 32) views on the absorption of electrolytes. Pirschle points out certain differences in the two points of view but at the same time recognizes a fundamental similarity, and regrets that Lundegårdh accepts as little of Osterhout's view as Osterhout does of Lundegårdh's. He further points out that the work of Osterhout, in developing thermodynamically exact deductions from experimental models which simulate the activity of large-celled algae, is directly related to the less deeply founded but broader concepts of Lundegårdh with respect to the metabolism and cell physiology of the higher plants.

Crafts & Broyer (17) discuss the problem of salt migration in roots as contrasted with the accumulation of salts by cells and suggest a mechanism to explain solute migration. The process of solute migration in roots appears to be definitely associated with the processes of accumulation by root cells. The mechanism of migration involves an activity gradient in the tissues which is conditioned by environmental differences imposed upon the tissues by their structure. Root anatomy

imposes an environmental difference upon tissues inside and outside of the endodermis and this environmental difference provides the physiological gradient essential to the mechanism proposed and discussed. It is suggested that "such a mechanism fits most of the data and is consistent with known root structure."

#### TRACE-ELEMENT NUTRITION

Information concerning the essentiality and importance of the so-called "trace" elements in plant nutrition continues to accumulate. While much of the earlier work dealing with the problems in this field is descriptive in nature, a marked change toward the more quantitative type of investigation has been in progress during the past two or three years.

Since extreme precautions are essential to avoid unknown sources of even the minutest traces of these elements in any studies dealing with their relations in plants, extended use has been made recently of improved methods of purifying salts and water (62, 68, 69) and of avoiding sources of contamination from culture equipment. Steinberg (67) estimates that an element may not be regarded as unessential if it is present in quantities greater than one part in a billion, although this degree of purity may not be attainable at present. This point as well as that relating to the absolute importance of these elements in mineral nutrition has recently been strongly emphasized in connection with the development and improvement of methods of growing isolated plant tissues *in vitro* (8, 60, 61, 80).

Many workers have reported symptoms of plant response associated with deficiency or excess of one or more trace elements. A list of references to such reports has been published (81). This list of references has recently been brought up to date and is now in press.

A comparison of the deficiency symptoms of certain trace elements with those produced by certain other essential elements has been reported by McMurtrey (45, 46). Typical pathological symptoms are evident on close examination of the root, stem, leaf or plant as a whole that serve to distinguish one deficiency from the other. It was observed that when more than one element is deficient, growth may be greatly reduced, but the evident symptoms are commonly those found to be typical for the element that is most deficient under the conditions. A key is given which is designed to aid in the identification of unknown deficiencies. The symptoms produced by deficiencies fall broadly into two groups. One group of symptoms includes those due

to lack of nitrogen, phosphorus, potassium and magnesium which appear to be readily mobile in the plant, and are localized on the older or lower leaves or are more or less general on the plant. Another group consists of those due to lack of calcium, boron, manganese, sulphur and iron. These elements are relatively immobile as judged by symptom manifestations which are localized in the terminal growth consisting of upper or bud leaves. The place of the trace elements in the mineral nutrition of plants has recently been discussed by Hoagland (26).

An interesting attempt has been made by Steinberg (70) to classify the biologically essential elements, both the trace elements and the major elements essential for growth, according to their atomic structure. He finds correlations between biological essentiality of chemical elements and atomic structure and suggests a form of periodic table based on "shell and sub-shell of transition, atomic number, and rank."

In an investigation on the role of molybdenum in the utilization of ammonium and nitrate nitrogen by *Aspergillus niger*, Steinberg (67) suggests that molybdenum is essential for the activation of the enzymes effective in the processes of nitrate reduction to ammonia which leads to the synthesis of amino acids and protein. He further suggests that biological specificity is a result of the chemical specificity of an element and such specificity presumably becomes more complete with increase in the number of reactions in which it simultaneously participates in the metabolism of the organism.

*Boron.*—The importance of boron in plant nutrition has been widely studied as is noted in a recent series of reference lists (18, 23, 81). Boron-deficiency symptoms in plants center particularly about injury to the meristems, and are evident very shortly after boron is omitted from the nutrient substrate regardless of the size or age of the plant. Recovery of a boron-deficient plant can be remarkably complete although the originally injured cells do not recover. On this point Brenchley (9) reported complete recovery and seed production during the second year, by sugar beets, following the occurrence of severe boron-deficiency symptoms during the first year.

Wadleigh (76) has shown from microchemical observations, that the development of boron-deficiency symptoms is paralleled by a progressive increase in acidity and ammonium nitrogen in scattered parenchymatous cells of stem tips of cotton. At the same time, as might be expected under these conditions, carbohydrates including reducing sugars, sucrose and starch accumulate. Tests also indicate

that a progressive degeneracy of the protoplasm takes place which results in disorganization of the affected cells.

Rehm (58) reports that the development of boron-deficiency symptoms occurs much more rapidly in plants grown in neutral or slightly acid nutrient media than in the more acid media. He finds that boron has an accelerating effect on the absorption of cations, such as potassium, and an inhibiting effect on the absorption of anions, but states that these effects are modified by the hydrogen ion concentration of the nutrient substrate. He suggests that the action of boron is a displacement of the isoelectric point of certain plasma colloids toward the acid side through the formation of complex boric acid compounds. However, his researches do not exclude the possibility that boron has definite functions other than its influence on salt absorption.

Phillips (54) measured the effect of the respiratory behavior of tomato fruits from plants grown in nutrient media at different boron concentration levels. His conclusions are interesting: Fruits from plants supplied with optimum boron concentrations yielded a low but steady rate of carbon dioxide output which appears to be conducive to good keeping qualities. Fruits from plants supplied with excess boron yielded steadier but higher rates of carbon dioxide output than those from plants grown with a deficient boron supply. Neither yielded as low a rate nor as steady an output of carbon dioxide as the fruits from plants grown with optimum boron.

*Zinc.*—Practically all of the evidence resulting from carefully carried out experimental investigations points to zinc as an essential element for the growth of both fungi and the higher plants. This is the general conclusion of Chandler (15) in his excellent review on the subject of "Zinc as a nutrient for plants," but he states that because of the very small amount of zinc required and its widespread presence as impurities it has required exceptional methods to hold the zinc supply, to plants in artificial media, low enough to prevent moderate growth. Although there has been some suggestion as to the role of zinc in plants no specific function of this element has been demonstrated.

The importance of zinc in the growth of lettuce and asparagus has been shown by Arnon (3) and its importance in the normal development of citrus has been emphasized by Haas (21) who reports data obtained in the study of several phases of the problem of the zinc relations in mottle-leaf of citrus. While zinc in appropriate concen-

trations is effective in preventing or in bringing about recovery from the mottle-leaf condition, the nature of its action is obscure. That its action is not independent but is influenced by the presence of other elements is indicated by the fact, as pointed out, that aluminum in appropriate concentration prevents or reduces the injury caused by excessive concentrations of zinc. An important feature of this work is a demonstration of the effective use of zinc in vegetative propagation by rooting of leafy-twigs cuttings of citrus.

Sinclair (66) investigated the diastase of orange leaves to determine whether diastatic analysis could be employed as a means of measuring the effect of treating citrus trees with solutions of zinc salts. He also deemed it essential to obtain information concerning the diastase reaction since zinc salts tend to precipitate proteins including diastatic enzymes and because of the physiological implications involved when mottled citrus leaves are sprayed with zinc sulphate. The results of this investigation bearing on the points in question were negative. Sinclair shows that in the presence of concentrations of zinc as high as 30 m. eq. per liter taka-diastase was capable of converting starch to glucose quantitatively, and that the diastase in macerated leaf tissue produced approximately equal quantities of reducing substances in concentrations of zinc ranging from 0 to 30 m. eq. per liter thus indicating the absence of any effect of zinc on the diastatic reaction.

*Copper.*—Whether copper is an essential element for the growth and development of many species of plants has perhaps not yet been definitely established. Its importance as a constituent of a growth substrate in minute traces has previously been reported, and again recently by Arnon (3); Saeger (62) states that *Spirodela polyrrhiza* responded favorably to a concentration of 0.1 part per billion added to a nutrient substrate prepared from highly purified salts. Copper added to a similar substrate at a concentration of 1.0 part per billion was clearly toxic, but this toxicity was overcome by the addition of iron, thus indicating an antagonistic relation between these two elements. Saeger failed to establish the essentiality of copper for the growth of this plant under the conditions of his experiments.

*Other elements.*—The importance, in the growth of plants, of elements other than those above mentioned has recently been reported by several investigators. While plants respond favorably toward these elements under specified conditions their essentiality for growth and development has not been definitely established. Lipman (36)

has reported on silicon, chlorine and aluminum; Scharrer & Schropp (63) on strontium and barium; and Steinberg (69) identified gallium as essential to the growth and development of *Aspergillus niger*. Warrington (77) investigated the effect of molybdenum on plants and finds that although it may not yet be regarded as being essential for growth it plays an important role, when present, in cytological as well as morphological behavior of the plant.

Selenium is of special interest because as Trelease & Trelease (75) observe it is the only element thus far known which plants absorb in sufficient quantities to become lethal to animals. The toxic properties of such plants have been described by Trelease & Trelease (74), Beath (5), Martin (47), and in a review by Trelease & Martin (73).

Several species of plants were found by Trelease & Trelease (75) and by Martin & Trelease (48) to accumulate selenium in the tissues in direct relation to the concentration of selenium (as selenite) and in inverse relation to the concentration of sulfate in the nutrient substrate. Growth was stimulated by the presence of low concentrations of selenium and it is suggested that selenium may be an essential element for the growth of some of these plants. The degree of toxicity to wheat plants caused by selenates has likewise been found by Hurd-Karrer (29) to vary inversely with the concentration of sulfate sulfur, but the toxicity caused by selenites was progressively more severe with increasing concentrations of sulfate in the nutrient substrate. An explanation to account for these phenomena is proposed and is based upon the fact that selenites are more easily reduced to elemental selenium in plant cells than are the selenates.

#### NITROGEN NUTRITION

Although the problems relating to nitrogen nutrition of the higher plants have held the attention of the plant investigator for many decades, interest in this field has shown no decline and today is keener than ever before. Some of the most important problems to be considered in large scale plant production are those involving the relation of nitrogen in its different forms to the general field of plant nutrition, growth, and yields of plant products. While great progress has been made in understanding the nitrogen relations in plants, the problems involving these relations still provide one of the more important and profitable fields of investigation.

In the light of the present confusion which surrounds the nutritional status of certain nitrogen compounds in normal plant life, much

quantitative information is still required before the network of reactions involved in nitrogen metabolism may be visualized in related sequence. That substantial progress along these lines has been made in recent years is well brought out in the excellent reviews of the work on the nitrogen metabolism of plants by McKee (43, 44) in which he affirms that the stages in the formation of protein are still obscure but concludes with a diagrammatic survey outlining the possible sequence in the nitrogen catabolism of the plant.

Nightingale (40) also indicates in a general way the metabolic sequence of organic nitrogenous materials in the plant as related to amino acid and carbohydrate transformations, and to the assimilation of inorganic nitrogen from external sources.

In this connection such thoroughgoing work as that reported by Petrie & Wood (53) and by Wood & Petrie (82) cannot but lead to a better understanding of the complex relationships involved in nitrogen metabolism. They visualize a network of reactions between the arrival of ammonia nitrogen (nitrate nitrogen is not here considered) in the leaves and the synthesis of proteins, and deal specifically, first, with the relation between the content of proteins, amino acids, and water in the leaves, and then with the interrelationships among the soluble nitrogen compounds, water, and the respiration rate. Space does not permit a detailed review of this work but several of the more pertinent conclusions may be stated. From the results of the work the authors conclude that the net rate of protein formation from amino acids decreases with reduction in the water content but it is not clear whether synthesis is decreased or hydrolysis is increased, or whether both are affected. The curve relating the content of residual amino nitrogen, and that relating amide nitrogen, with ammonia nitrogen content are concave to the axis representing the content of ammonia nitrogen. A plausible explanation of the nature of these curves is offered. The respiration rate was found to increase as the water content of the leaves decreased to a certain level; further decrease in water content resulted in a lowered respiration rate. It is noteworthy that no significant regression coefficients could be obtained for the respiration rate.

In a study of ammonium nutrition in relation to metabolism and growth, Burkhart (13) made use of etiolated seedlings of several species, assuming that such material would be suitable for the purpose of obtaining information concerning the intermediate products of protein metabolism, since protein decomposition is favored and its regeneration hindered in the absence of light because of the depletion of



available carbohydrates. An attempt is made to throw additional light upon the fundamental question of what determines the rate of ammonium absorption by seedlings and to what extent and under what conditions the ammonium is utilized in organic synthesis. As a result of these investigations certain trends were observed in the various species and at least three distinct internal conditions or stages were recognized with intergradations directly associated with the response of the seedlings to ammonium nutrition.

In the early stages of growth when the tissue content of available carbohydrates is high, rapid absorption and utilization of ammonium is associated with marked depletion of sugars resulting in protein synthesis and rapid growth.

In the intermediate stages with available carbohydrates partially depleted, protein hydrolysis occurs and amides accumulate. During this period ammonium has little effect upon growth. When available carbohydrates have become seriously depleted, ammonium is neither utilized nor absorbed, but accumulates as the result of the breakdown of nitrogenous organic compounds, and excretion of nitrogen into the substrate occurs. During this period both proteins and amides are decomposed and an accumulation of amino nitrogen may or may not occur. So-called "ammonium injury" is considered to be the result of severe carbohydrate depletion and not due to the accumulation of ammonium in the tissues.

Keen interest still exists in the relative nutritional effectiveness of ammonium and nitrate nitrogen, and particularly is the interest keen in problems concerned with the relative effects of these two forms upon the nature and course of the processes involved in nitrogen absorption, and the transformations of the inorganic to the organic forms of nitrogen.

Thus Sideris *et al.* (64, 65) finds that ammonium assimilation by the roots of several species is very rapid if not instantaneous, a conclusion arrived at by other investigators (28) also. The immediate stable products of ammonium assimilation found in appreciable amounts in the tissues are amino acids, asparagine, and glutamine, but the various reactions which precede the synthesis of these compounds are unknown. On the other hand, nitrate nitrogen is not assimilated as rapidly as ammonium nitrogen, nor are the various fractions of soluble organic nitrogen in the tissues as high in quantity following nitrate assimilation as they are following ammonium assimilation. However, the fraction of insoluble (protein) nitrogen is

higher in roots of the plants grown with nitrate than in those grown with ammonium as the source of nitrogen. A tentative explanation of this phenomenon is offered.

Ludwig (37) reports that both ammonium and nitrate nitrogen, as well as many forms of organic nitrogen, are readily assimilable by a green alga, and the results given are confirmatory of the idea that in nature green plants are not necessarily dependent upon nitrification to render organic nitrogen assimilable. Similar studies (1, 22) dealing with the nitrogen nutrition of the higher green plants have recently been reported.

Arnon (2) investigated the ammonium and nitrate nitrogen nutrition of barley in relation to several variable external factors, and discusses some general views regarding the possible functions of "catalytic" metals and aëration of the roots in relation to the utilization of these two forms of nitrogen. A significant point brought out is the fact that the growth of the plants was strongly but favorably affected by either forced aëration of the liquid substrate or by the addition to it of manganese, copper, or certain other metals without forced aëration.

The importance of considering the interaction of the variable factors concerned in any study of nutritional relationships has been strongly emphasized by the work of White (78, 79) who investigated the interaction of nitrogen and light intensity in relation to growth and assimilation in *Lemna*. It was found that the characteristic symptoms of nitrogen deficiency are intensified by increase of light intensity with constant nitrogen supply, and those of nitrate excess by decrease of light intensity with constant nitrate supply. It is concluded that maximal growth is obtainable only when the factors of light and nitrogen are in suitable ratio. To retain this condition of balance, increase or decrease of one factor must be accompanied by corresponding increase or decrease of the other factor. A general theory based on this principle is proposed:

Whether increased growth occurs or not is the resultant of a positive effect due to the increase of the major factor and a negative effect due to the greater relative deficiency of all other factors and is determined in any particular case by the relative importance of the factors at work.

In the same manner Gregory & Sen (20) have investigated the relation of the respiration rate to the carbohydrate and the nitrogen metabolism as determined by nitrogen and potassium deficiency. The results are examined statistically and are discussed in relation to the

source of carbon dioxide in aërobic respiration. In this category also may be included the extensive work of Richards (59) on the relation of the respiration rate to the carbohydrate and nitrogen metabolism of barley as determined by the phosphorus and potassium supply. The hypothesis is suggested that in the normal plant growing under balanced nutrient conditions efficiency of respiration in this respect is maximal. Space does not permit even brief consideration of other important papers (7, 14, 19, 51) dealing with the more practical aspects of this subject which have appeared during the past two years.

The literature bearing upon this general question of nitrogen nutrition in relation to the metabolic status of the plant is quite voluminous, as is indicated by Nightingale's (41) recent comprehensive review on the subject, yet the large number of papers appearing at frequent intervals which deal with these fundamental problems indicates the keen interest which is maintained in this important phase of general plant science.

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# GROWTH HORMONES IN THE HIGHER PLANTS

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This report can be considered as a supplement to the monograph by Went & Thimann. Although mainly the literature of 1938 is reviewed, some earlier publications have also been included. Other recent reviews dealing with the subject are Thimann & Bonner, Bonner (2), and Söding (4).

This year also the report of the growth-hormone conference, held in Paris in 1937 under the auspices of the International Union of Biological Sciences, appeared. Unfortunately, the changes made in the submitted papers in the course of editing altered, in certain cases, the meaning of the author, as will be seen by comparing the résumé of Bouillenne's paper ("Etudes et recherches sur les phytohormones," *Inst. Intern. Coop. Intellect.*, 1938, p. 65), with the original (Bouillenne, p. 344).

The proposals for the nomenclature of the growth substances of plants are simple: two groups are recognized, the auxin and the bios group. Within each group a distinction is made: (a) phytohormones, or substances made by the organism itself; and (b) substances with similar effect, and specific food factors. This shows that auxin is now generally accepted as a generic physiological name as had been done for some time in America. Following the proposal of Went & Thimann, the name auxin will be used for substances causing growth by cell elongation as measured in the *Avena* test; auxin-a and auxin-b are specific chemical names for the substances isolated by Kögl and collaborators. The distinction between phytohormones and non-native substances with a similar effect may be important theoretically, but as yet too little is known to use the distinction in practice.

Last year also the first patents were issued covering the use of growth-promoting substances. Since the practical application of these substances to root formation was the result of the concerted action of many different investigators, it is misleading that the patents (U.S.P. 2,129,598-2,129,601) bear only the names of Zimmerman and Hitchcock.

## AUXIN AND GROWTH IN LENGTH

*Technique.*—Many papers appeared describing methods for auxin determination. The *Avena* test [Schneider & Went; Söding (2); Thimann & Schneider (1)] and the pea test [van Overbeek & Went; Jost; Thimann & Schneider (2)] were more closely analyzed as also the determination of auxin in lanolin pastes (Linser). It has been shown that neither the original explanation nor that of van Overbeek & Went holds for the pea test. But no hypothesis explaining all peculiarities of this test has been published as yet. However, all authors have agreed that the curvatures are due to the increased growth of the intact side of the split stems. Skoog (1) described a new deseeded *Avena* test, which allows the determination of much smaller auxin concentrations than the standard *Avena* test (Went & Thimann). Söding (2) mentioned that the *Cephalaria* test, although sometimes extremely sensitive, cannot be used for quantitative auxin determinations. The auxin extraction also has been improved by Boysen-Jensen (1), by DuBuy and especially by van Overbeek (2, 5). Methods for measuring auxin concentration by observing its effect on straight growth were modified by Scheer, by Granick & Dunham and by Weintraub. The methods employed at the Boyce Thompson Institute to determine the effectiveness of growth-promoting substances have been worked out on a more quantitative basis [Zimmerman & Hitchcock (2); Hitchcock & Zimmerman].

About the chemical nature of the auxin in the higher plants no new data have been published. Lefèvre determined colorimetrically the amount of indole compounds present in expressed or extracted juice from different plants, but this has no bearing on their auxin content. He finds more than one part indole in  $10^5$  juice, whereas it is known from physiological experiments that the auxin content of plants generally not exceeds one part in  $10^8$  [van Overbeek (5)].

*Auxins and growth.*—Much more evidence has been collected concerning the occurrence of auxin and its role in growth and correlation in the plant. For the most part this evidence has confirmed existing views. Avery, Burkholder & Creighton (1) and Söding (3) have investigated the auxin balance of shoots of the horse chestnut (*Aesculus*), apple (*Malus*) and *Heliopsis*. The buds of the first two species begin to give off auxin just before they start to develop in spring. The auxin production increases until just before the most rapid elongation of the shoot and then decreases. Cambial growth follows closely the auxin production in the stem. [For the effect of

auxin on cambial activity cf. Söding (1).] In *Heliopsis* the young plants contain the most auxin; the auxin level gradually drops upon aging. Auxin production occurs mainly in growing buds, flower buds and young leaves. (See also Goodwin.)

In *Pulsatilla* Kaupp found that the growth rate of the flower stalks was directly proportional to the amount of auxin which could be diffused out of them. On the other hand van Overbeek (5) found the highest growth rate in corn seedlings correlated with the lowest auxin content. For this he offers two explanations: (1) the auxin content is low because so much is used up in growth; and (2) the combination of auxin and food factor is most favorable for growth. Avery & LaRue measured growth in excised *Avena* coleoptiles, and found growth to continue for six days after the last auxin could be diffused out of the tips. Therefore they question the old rule: without auxin no growth. In this connection one should remember, however, that in *Avena* there is a relation between extractable auxin and growth, and not between diffusible auxin and growth [Went (4)].

Sereiskij & Sludskaja observed that the growth of untreated and yarovised wheat seedlings is directly proportional to the auxin content of the seed, which increases upon cold treatment. Bennett & Skoog reported that auxin precursor can be obtained from the buds of an apple tree about a month before they start to develop rapidly, and that shortly before the breaking of the buds free auxin also appears. Voss proposed that the reason no auxin can be obtained from the scutellum of germinating grass seeds lies in the fact that the active auxin from the endosperm is transformed into an inactive form in the scutellum which is subsequently reactivated by the tip. A simpler assumption is, however, that only inactive auxin precursor, which is already present in the endosperm, moves through the scutellum. Van Overbeek (3) extended his determinations of auxin production in genetic dwarf races of corn to five other dwarf types. In all of them he could confirm his former results that auxin production is somewhat smaller in dwarfs than in normal plants; however, through excessive destruction little or no auxin reaches the growing zones of the dwarfs. The investigation of *Agrostemma* seedlings (Höfner) showed that auxin production resembles that of *Helianthus* and *Raphanus*. For *Lupinus* the existing view that auxin is produced everywhere in the plant where growth occurs, with hardly any transport, was confirmed by Jahnel (1). In *Avena* it was found that soon after the roots were removed, the auxin production was decreased [van Over-



beek (1)]. In *Raphanus* seedlings Gorter & Funke found that when grown in a moist atmosphere less auxin was produced than in dry surroundings, although the total growth was greater. Some investigations have shown that nutritional deficiencies in *Helianthus*, *Nicotiana* [Avery, Burkholder & Creighton (3)] and *Raphanus* (Gorter) result in a decreased or, in the case of nitrogen deficiency, even completely interrupted auxin production. The decrease in production of auxin precedes any measurable inhibition of growth. In degenerating potatoes a slight decrease in auxin content was found [Jahnel (2)]. Correspondingly such potatoes gave a positive response to application of indoleacetic acid, which did not occur in normal potatoes (Ramshorn).

Effects of applied growth substances on the growth of normal plants have been described by various investigators. In most cases no marked increase in growth has been found in the lower concentrations when applied to the root system (Marmer; Greenfield). However, Pearse observed a positive growth response after spraying tomato plants with phenylacetic and indolebutyric acid. Pfahler increased the growth of *Epilobium* and other plants by application of indoleacetic acid to the tip of the stem with a small brush.

*Effect of illumination.*—The effect of light on auxin formation and content is complex. In the long run light is essential for the formation of auxin in the higher plants. This was confirmed again by Avery, Burkholder & Creighton (4) for tobacco (*Nicotiana*) plants, which lose their auxin in from two to ten days when kept in darkness. After illumination auxin reappears within ten hours. Its formation is a function of the light intensity and the duration of illumination. Cajlachjan & Zdanova (1) also found that when plants are illuminated for only eight hours daily they invariably produce less auxin than when grown under "long day" conditions. This is independent of their photoperiodic response in flowering. Segelitz found that *Zea* roots grown *in vitro* form auxin, especially when grown in the light.

The more direct effect of light upon auxin is an inactivation (Burkholder & Johnson; Koningsberger & Verkaaik), especially when the auxin is inside the plant tissues. Kögl has suggested that this inactivation is due to the light-sensitivity of the auxin- $\alpha$ -lactone, but direct proof of this is still lacking. *In vitro* the lactone is inactivated by the shorter ultraviolet radiation only (C. Koningsberger) but in the intact plant visible radiation is effective.

*Analysis of the auxin effect.*—A number of papers have been concerned with the mechanism of the action of auxin inside the cell. The first observable effect, appearing a few minutes after auxin application, is an acceleration of protoplasmic streaming (Sweeney & Thimann). However, this effect does not seem to be correlated with the growth reaction [Went (4)], as DuBuy & Olson suppose.

Inside the cell the auxin reaction is connected in some way with the available sugar. Schneider showed that when *Avena* coleoptile cylinders are grown in a solution containing sucrose and indoleacetic acid, the growth is proportional to the product of the logarithm of the sugar and the logarithm of the indoleacetic acid concentrations. He definitely proved that sugar is one of the food factors required for the growth reaction. The presence of salts also affects growth, but the exact way in which the salts act is still very obscure inasmuch as different investigators have obtained contradictory results: Wuhrmann having obtained a decrease in growth rate; Borris and Thimann & Schneider (1) having obtained an increase.

W. S. Stewart was unable to find a specific effect of indoleacetic acid on the extensibility of dead cell walls consisting of cellulose. Ruge (1, 2, 3), however, supposes that this effect occurs in the intermicellar substance, increasing its plasticity without the intermediary of the protoplasm. The first measurable changes in protoplasmic properties, namely increased viscosity and decreased permeability, he found only after eighteen hours (Thimann & Sweeney observed an effect within five minutes!). This shows that these changes in protoplasmic properties have nothing to do with growth. Ruge (3) also showed that, in *Helianthus*, cell growth may or may not be accompanied by laying down of new cell wall, which he considers as intussusception during growth and apposition when growth has ceased through decapitation.

The investigations of Frey-Wyssling & Schoch-Bodmer, Wuhrmann & Meyer, and van Iterson have furnished further proof that elongation of cells is closely correlated with a "tubular" structure of the primary cell wall (orientation of the cellulose micelles more or less perpendicular to the long axis of the cell). Preston claimed that in the *Avena* coleoptile this arrangement is spiral rather than tubular. According to Wergin no reorientation of the micelles occurs after completion of growth in length of cotton hairs, but the secondary cell wall, which forms subsequently, has fiber-structure. This soon changes the original negative anisotropy of the cell into positive anisotropy.

Another effect of indoleacetic acid on plants has been described by Hamner & Mitchell, Czaja, N. W. Stuart, and Alexander, namely that treated plants gain more in dry matter than untreated plants during the six days after treatment. This gain is most noticeable near the place of application and is mainly due to increase in cell wall material. The effect is especially marked with higher concentrations of indoleacetic acid, which cause inhibition of lateral bud growth. For this reason buds have to be removed from the treated and the control plants before application of the hormone so as to obtain comparable results.

*Anatomical effects.*—The morphological and anatomical effects of auxin have been described, especially for the swellings which appear when high concentrations of indoleacetic acid are applied, for example to decapitated stems (Borthwick, Hamner & Parker; Goldberg; Hamner; Hamner & Kraus; Harrison; Kraus, Brown & Hamner; Link, Wilcox & Link; Scott). The effect seems to be similar in most plants: proliferation of parenchyma into a large callus; formation of distinct meristematic regions, mostly in connection with or near pre-existing vascular elements (endodermis, pericycle); and differentiation of root primordia in these meristematic regions. No consistent quantitative differences in response were found with different growth-promoting substances, indicating that their mode of action is similar. These swellings resemble very much those produced by *Bacterium tumefaciens* (crown gall), so that many investigators have attributed the crown-gall swellings to auxins produced by the bacterium (Brown & Gardner; Link & Wilcox; Locke, Riker & Duggar). But recently the latter authors have shown that other factors must be involved as well, since nonpathogenic bacteria formed as much growth-promoting substance as pathogenic strains.

#### GROWTH AS A CHEMICAL REACTION

Following the discovery of the growth-promoting activity of indoleacetic acid (Kögl, Haagen-Smit & Erxleben) a number of new substances with the same type of activity have been found [Kögl & Kostermans; Haagen-Smit & Went; Thimann (1); Hitchcock; Zimmerman & Wilcoxon, etc.]. Since at first sight no close chemical relationship between the active compounds was evident and the relative activities were so different, the plant-growth substances were classed as stimulants [Fitting; Boysen-Jensen (2); Avery; Hitchcock & Zimmerman; Avery & LaRue].

However, Thimann (1) and Haagen-Smit & Went had already pointed out that under the proper conditions the molar activity of different growth-promoting substances is of the same order of magnitude. This fact has been further stressed by Went & Thimann, van Overbeek & Went, Koepfli, Thimann & Went. Recently D. Bonner (2) clearly showed that even for substances which seemed much less active in the standard pea test, the molar growth activity is the same as that of indoleacetic acid. *Ciscinnamic* acid has, uncorrected, a molar growth activity only a fifth as great as indoleacetic acid. It is a much stronger acid; therefore at the same concentration inside the cell a much smaller fraction of undissociated molecules will be present. If the activity is calculated in terms of these undissociated molecules, the molar growth activities of indoleacetic and *ciscinnamic* acid are the same. After the elimination of still other complicating factors the growth activity of phenylacetic acid was found to be not less than 17 per cent of that of indoleacetic acid.

Since all these studies have indicated that the growth reaction is a reaction with a stoichiometric relationship between growth-promoting substance and growth produced, the minimal structural requirements of a molecule for growth-promoting activity have been determined by Koepfli, Thimann & Went. These workers have found that a compound with an unsaturated ring-system, with a side chain of at least 2 C-atoms, which contains a carboxyl group with definite space relationship to the ring-double bond, is required for growth activity. Their general conclusion was that physiological activity is not necessarily correlated with a definite nucleus, but rather with a specific molecular arrangement which may be present in compounds with ring-systems of very different types.

The penetration of indoleacetic acid into *Nitella* cells was found to be greatest at a low pH, its pH-dependence following a curve closely resembling the dissociation curve of indoleacetic acid (Albaum, Kaiser & Nestler). This is clear evidence that only the undissociated molecules enter the cell. A new analysis of the pH-dependence of growth of sections of *Avena* coleoptile [van Santen; D. Bonner (2)] has confirmed J. Bonner's conclusion, that auxin is active only in its undissociated form. This is independent of the penetration. Since only the undissociated molecules cause growth, the pH affects growth, when auxins are applied, in at least two ways: it changes the number of undissociated molecules that enter the cell, and alters the dissociation of the acid inside the cell.

In this connection it should be mentioned that some investigators have stressed the greater activity of the growth substances when supplied as salts rather than as free acids [Zimmerman & Hitchcock (2); Avery, Burkholder & Creighton (2)]. From the foregoing considerations these results would not be expected, since the free acid would penetrate more rapidly and once inside the cell no differences in activity would be expected due to the buffering action of the cell contents. And indeed, upon closer analysis no such differences have been found [D. Bonner (1); Scheer; Thimann & Schneider (1)].

*Auxin transport.*—Concerning the transport of the growth substances many new data have been accumulated. Except in a few investigations, in which the work was not done with low physiological concentrations, the polar movement of auxin inside the living plant has been found. Skoog (2) has shown that when indoleacetic acid is applied to the root systems of tomato and squash in concentrations higher than the equivalent auxin concentrations occurring inside the plant, it is taken up and transported in the transpiration stream. As soon as it leaves the xylem elements, however, it is subjected to the normal basipetal polar transport. In untreated plants no auxin was found in the xylem exudate. This indicates that normally no transport of auxin occurs in the transpiration stream. In the contents of the phloem of trees Huber, Schmidt & Jahnelt demonstrated the presence of auxin and Söding (2) found that especially the cambium is rich in auxin.

Clark (1, 2, 3) in extensive studies has shown that the bioelectric potentials, which can be measured in *Avena* coleoptiles or other plant organs with electrometer or galvanometer, are not correlated with the polar auxin transport, as supposed by Went. Experiments on the effect of applied potentials on growth led Cholodny & Sankewitsch to the same conclusion. Clark (3) also in a direct experiment demonstrated the independence of auxin transport and protoplasmic streaming. From circumstantial evidence DuBuy & Olson deduced a parallelism between the effects of applied potentials on auxin transport and protoplasmic streaming.

#### GROWTH FACTORS SUPPLEMENTARY TO AUXIN

From the very beginning it was realized that the auxin acted only in conjunction with other factors to cause growth. Some of them were required as sources of energy, some of them were hormones themselves. For root formation this was shown to be the case

(Went & Thimann; Went, Bonner & Warner). Schneider identified sugar as one of the food factors necessary for growth in length of the *Avena* coleoptile. Laibach & Fischnich stressed the importance of assimilation for growth. Went (1) had already pointed out that the manifold activities of the auxins could be explained only by the co-operation of auxin with other as yet unidentified growth factors, calines. In later papers he has elaborated this view (2, 3) and collected more evidence for their existence. Bouillenne reached the same conclusion in regard to root formation. Michener (2) also presented evidence that auxin swellings require a factor coming from the roots, in addition to the effect of auxin.

#### AUXIN AND ROOT FORMATION

The most important practical applications of the auxins lie in the field of root formation. A large number of papers have appeared describing the effect of indoleacetic acid and similar compounds on root formation in a large number of different varieties of plants. Only a few of these will be mentioned: Almeida; Cooper & Manton; Cooper & Went; Evenari & Konis; Gocolasvili & Maximov; Hubert & Beke; Laibach; van der Lek & Krijthe; Pearse (2); Stoutemyer (2); Tincker.

Most investigators have reported that treatment with auxins increases the percentage of cuttings rooted and the number of roots produced per cutting, as well as reduces the time necessary for rooting. The substances reported to be the most active are indoleacetic acid, indole-butyric acid and naphthaleneacetic acid. In general the concentrations applied have been of the order of .01 to .5 mg. per cc. Some authors have pointed out the importance of adding sugar to the auxin (Evenari & Konis; Fischnich). Other authors have reported that an after-treatment with thiamin (vitamin B<sub>1</sub>) brings out roots when a single treatment with indoleacetic acid is ineffective (Went, Bonner & Warner). The reason for the effectiveness of vitamin B<sub>1</sub> will be mentioned below. Hellings pointed out again the strong polarity which exists in the case of root formation, and like Cooper has assumed the phloem to be the tissue through which auxin is transported. The anatomy of root formation under the influence of the auxins has been described by Dorn, Hellings, Pfeiffer, Scott, and Stoutemyer (1).

There are three opinions concerning the effectiveness of auxin in root formation. Some workers (Bouillenne) question the necessity

of the presence of auxins for root formation because in their experiments root formation occurred without indoleacetic acid application. Other investigators maintain that the auxins themselves are the specific agents for causing root formation. These investigators have found that in the plants they have tested the application of indole compounds always led to root formation [Hitchcock & Zimmerman; Pearse (2); Hellinga]. Finally, other workers hold that the auxins are but one of the many factors necessary for root formation. In this connection, the experiments of Cooper should be mentioned first. Cooper found that although the internal indoleacetic acid concentration was identical in both lemon and apple cuttings after identical treatment with indoleacetic acid, the lemon cuttings rooted very well, whereas the apple cuttings showed no sign of rooting. This indicates that apple cuttings are deficient in another factor required for root formation. In other experiments he showed that a basal indoleacetic acid treatment of definite concentration and duration greatly decreased the ability of the rest of the lemon cutting to form roots although treated immediately thereafter with indoleacetic acid. This was explained by assuming that another factor required for root formation (rhizocaline) disappeared from the cutting during root formation. Experiments of Went (2) form a counterpart to those of Cooper. In pea seedlings it was found that a limited amount of a specific root forming factor was present which was redistributed inside the stem by applying indoleacetic acid.

A number of authors have criticized these conclusions. They base their criticisms mainly on the negative results they have obtained [Hellinga; Pearse (2); Hitchcock & Zimmerman]. However, in most cases these authors have not varied the conditions sufficiently in their experiments to warrant their conclusions.

Traub has reported that a large number of compounds, lacking growth activity, were effective in causing root formation on *Passiflora* cuttings. This shows that growth activity and root-forming activity are due to different properties of the molecule, causing different responses in the plant. These substances, just as ethylene, are only effective when the cuttings contain a sufficient amount of auxin of their own [Michener (1)].

#### AUXIN AND BUD GROWTH

Inhibition of the outgrowth of lateral buds on shoots was known to depend on the auxin formed by the apical bud. Thus the same

auxin which is indispensable for growth in length of the main branch inhibits the growth of the undeveloped lateral shoots. Münch applied the knowledge of the hormonal inhibition to explain the general shape and branching of trees. He came, however, to the remarkable conclusion that this co-ordination by auxin is "a reckless war of all against all," since each growing shoot inhibits the growth of all surrounding ones, which it tries to outgrow, to poison, etc. Münch also supposed that the geotropic and epinastic reactions of lateral shoots, especially in conifers, are due to the action of the auxin formed in main and lateral shoots. Direct experiments on the role of auxin in bud inhibition have been reported by Dostal, Snow (1, 2), Thimann (2), Ferman, and van Overbeek (5). The two latter workers have analyzed the auxin content of the stem and bud by the extraction method and have established that the auxin content of the inhibited bud is lower than that of the apical or inhibiting bud, but that it may become very high after apical auxin application. Van Overbeek (5) found that immediately after decapitation the auxin content of the lateral buds increases, even of the lower ones, which presently will be inhibited by those developing above them. He came to the conclusion that a high auxin content of the stem adjoining the lateral bud causes its growth inhibition, by the effect of this auxin on the movement of bud growth factors. Ferman assumed that the auxin production causes the movement of the auxin precursor toward the centers of production, thus preventing it from reaching the non-growing buds, which thus continue to be inhibited. Snow (1) assumed that the auxin on its path downward in the stem releases an inhibiting substance, which prevents all lower buds from growing out. Finally Thimann (2) presented the hypothesis that bud growth is stimulated by low, and inhibited by higher auxin concentrations, as has been shown in root growth. The normally occurring auxin in the stem, which accelerates cell elongation, would be already strongly inhibiting for lateral buds. In fern prothallia a similar inhibition of adventitious outgrowths is exerted by the apex and this action can be replaced by applied indoleacetic acid (Albaum).

Reports on the action of auxin on bud formation also have been published. Beal has reported that the application of indoleacetic acid paste to the cut surface of a decapitated *Lilium Harrisii* resulted in bud formation and development at the cut surface. Prevot, Goldberg, and Link & Eggers have noted the same phenomenon in *Begonia Rex*, cabbage and flax. Greenleaf found that this type of treatment



induced the formation of polyploid buds between normal ones on tobacco stems, causing a response similar to that of colchicine. Tutschova described the formation of fasciated cotyledonary buds in *Phaseolus* after indoleacetic acid application. Other malformations were obtained by M. Snow & R. Snow by applying indoleacetic acid to the growing point of *Epilobium* or *Lupinus*. The primordia subsequently arising were abnormally large, or displaced, thus altering the phyllotaxis. Dostal & Hosek described how applied indoleacetic acid will change the nature of the lateral shoots in *Circaea*. Apical shoots which normally would develop into flower shoots become bulbils or stolons (runners), whereas prospective stolons may develop into upright leafy shoots.

#### PARTHENOCARPY

Parthenocarpy, the development of fruits without the accompanying seed development, can be induced by application of auxin-like substances to the unfertilized ovary of many plants. Gustafson (1) induced the development of normal-sized fruits of unfertilized tobacco, tomato, squash and other ovaries by applying indoleacetic, indolepropionic, indolebutyric and phenylacetic acid to the cut style. Gardner & Marth obtained similar results by spraying holly, strawberries and other plants with solutions of some of the same and similar compounds. However, all authors agree that with certain plants parthenocarpy cannot be so easily induced. Gardner & Kraus report that the general anatomy of the parthenocarpic and normally pollinated holly fruits is approximately the same, except, of course, the lack of development of embryo and integuments. Recently Se-reiskij induced parthenocarpy in pears. Gustafson (2) has added to his former results, by testing many other compounds. It is interesting that certain compounds, such as pyrrole-alpha-carboxylic acid which does not have any cell-elongation (auxin) activity, induce parthenocarpy. This indicates that parthenocarpic development is not a result of the growth-promoting properties of the compounds used.

By applying indoleacetic acid or similar compounds to seeds (Shibuya; Thimann & Lane; Cholodny; Amlong & Naundorf) an increased germination and a better further growth, or even yarovisation, have been obtained in many cases. Other authors have reported no effect [Veh & Söding, Cajlachjan & Zdanova (2)]. Dormant turions of *Stratiotes* will sprout prematurely when treated with indoleacetic acid (Vegis), but in dormant potatoes it has the opposite

effect (Guthrie, but see also Ramshorn). This means that no general rules for the effect of auxin on germination can be given. It is only when the auxin content of the seed is very low that it may be limiting the germination (Sereiskij & Sludskaja), but every case will have to be considered separately.

#### TROPISMS

The mechanism of the geotropic curvature was studied in lazy corn, an ageotropic race, by van Overbeek (4), who found that it was due to a lack of the normal redistribution of auxin under the influence of gravity (which normally moves to the lower side of a horizontally placed stem). In roots Syre did not find a parallelism between redistribution of starch grains and geotropism, which is required by the statolith theory of Haberlandt. He also showed that erythrosin treatment, which abolishes geotropic sensitivity, does not destroy the auxin inside the roots, as had been assumed by Boysen Jensen, but only inactivates auxin at the cut surface. Zimmerman & Hitchcock (1, 3) investigated the geotropic and phototropic curvatures of tomato plants and accepted the Cholodny-Went theory for these phenomena, namely that these curvatures are due to unequal distribution of growth-promoting substance inside the plant. Synthetic compounds may take the place of the natural hormones which disappear in prolonged darkness. When they applied high concentrations of growth substances, they claim to have obtained positive geotropic curvatures of shoots, a reversal of the normal negative geotropic response. However, here they mistook the passive sagging of the stems due to their increased plasticity for a tropistic response. Oortwijn Botjes showed that prolonged darkness first opposes geotropic response by the disappearance of auxin from the stems, and later by abolishing the perception of gravity. Witsch found that from one to three hours after auxin application plagiotropic *Tradescantia* shoots and positively geotropic roots become negatively geotropic.

Burkholder & Johnston's, and Koningsberger & Verkaaik's experiments stress the importance of the part that the destruction of auxin by light plays in the production of phototropic curvatures, a fact established by van Overbeek. The reaction to indoleacetic acid of tendrils was found to be similar to the haptotropic response: the growth of the convex side is increased, but on the concave side a growth inhibition is induced (Boresch).

## ROOT GROWTH

So far we have not discussed the growth of roots. The earliest known effect of auxin on roots was a growth inhibition. But at very low auxin concentrations root growth is stimulated (Amlong). There is a controversy as to whether the auxin present in the root tip is produced there. Although Fiedler could not obtain auxin from root tips grown *in vitro* for more than one day, Nagao (1, 2) found that from *Helianthus*, *Pisum* and *Zea* root tips auxin can be extracted after from two to six days of cultivation on a nutrient medium. Van Overbeek & Bonner carried on these experiments for a longer time and found a considerable amount of auxin in pea root tips cultivated for three weeks in nutrient medium, although the total amount was less than in the original tip. Van Raalte claimed that more auxin would diffuse out of root tips than could be extracted from them, indicating synthesis of auxin in the root tip.

Although auxin plays an important part in regulation of root growth, it can probably not be considered as a specific root growth factor, as it is for stem growth. Bonner (1) and Robbins & Bartley (1, 2) recognized that vitamin B<sub>1</sub> was one of the main specific factors required for root growth, which was confirmed by White (2) who also suggested [White (1)] the significance of amino acids for the growth of tomato roots. We may conclude that vitamin B<sub>1</sub> is a growth hormone for roots and is supplied by the parts of the plant above the ground. Recently also nicotinic acid was shown to be an essential growth factor for pea roots [Bonner (4), Addicott & Bonner]. When fed along with vitamin B<sub>1</sub>, sugars and salts it is able to give sustained growth of pea roots at a maximal rate for over two months. In this connection it should be stressed that no amino acids are required so that in the presence of vitamin B<sub>1</sub> and nicotinic acid pea root cells are able to synthesize amino acids from sugar and nitrate. This had been inferred already from other experiments, but these experiments definitely prove it. Further studies of the technique of root-tip growth have been published by Robbins & White, Robbins & Schmidt, White (3), and Delarge.

Very interesting are the results obtained concerning the molecular specificity of vitamin B<sub>1</sub> for root growth. Whereas in animals only one thiamin is active, roots are able to use its component parts, pyrimidine and thiazole [Robbins & Bartley (2), Bonner (3)]. The specific pyrimidine present in the vitamin molecule may be slightly altered without loss of activity. The same is true for the thiazole, in

which the hydroxyl group may be shifted from one C-atom to another in the side chain without destroying its activity as a vitamin [Bonner (3)]. The pea root is even able to utilize intermediates of the thiazole (thioformamide and acetopropyl alcohol) instead of the thiazole itself. That the pea root can effect a ring closure from these two acyclic compounds was proved directly by extracting the pea roots grown in a mixture of pyrimidine, thioformamide and acetopropyl alcohol, and testing this extract by the *Phycomyces* assay (Bonner & Buchman).

The results of the experiments of Bonner & Greene show that through its influence on root growth vitamin B<sub>1</sub> may increase the total growth of plants in sand culture. They conclude that vitamin B<sub>1</sub> may be an effective component of manure.

#### HORMONES AND FLOWER FORMATION

The work done in previous years has made it clear that flower formation in plants is governed by a hormone-like factor. The evidence for this has been based mainly on the induction of flower formation on plants, which by themselves could not form flowers under the experimental conditions. Two conditions were investigated: flowering of biennial plants in the first year, by grafting a shoot of the annual variety on it [Melchers (1)]; or flower formation on "short day" plants during a "long day" treatment by keeping one shoot under "short day" conditions; or by grafting a shoot of a plant previously treated with "short day" on the "long day" plant [Cajlachjan (2)]. In these cases a transmission of the flowering impulse was carried over from the flowering to the nonflowering graft-partner.

Cajlachjan & Zdanova (1, 2) proved that flower formation was not correlated with auxin formation either in long or short day plants, or in yarovised wheat or oats. Kuijper & Schuurman repeated the original experiment on the transport of flower-forming substances in the soybean. Hamner & Bonner in an extensive study of the photoperiodic behavior of the cocklebur (*Xanthium*) brought more direct evidence for the existence of a flower-forming hormone, and also studied another phenomenon, photoperiodic induction, which also is caused by a transportable agent. Their experiments clearly show that flower formation in the cocklebur is a response to a long dark period, and not the result of short days. Some further experiments of Melchers (2) indicate that nonflowering plants can induce flower

formation when grafted on the nonflowering stocks. This indicates that for flower formation a combination of hormonal factors is necessary, any one of which may be lacking in a given variety of plant.

Auxins and related compounds are not the only substances that cause growth of plant cells. Traumatin, probably identical with Haberlandt's wound hormone, is an important agent. Further reports on its purification were published by Bonner & English.

No mention will be made of the experiments done with animal hormones on plant growth; the reader is referred to the review of Zollikofer.

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## ANIMAL POISONS

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The capacity to elaborate poisons is manifested in a variety of forms and in many species throughout the animal kingdom from the monotremes to the protozoa. In some species, for example in salamanders, newts, frogs and toads, it has a purely passive defensive function; the venom glands are distributed in the skin and there is no special mechanism for inoculation. In others the venom apparatus is a weapon of offence and defence; the hind limbs of the male platypus are armed each with a canalised spur connected with a venom gland on the back of the thigh; many of the poisonous fishes have a venom gland situated at the base of a spine. In a wide range of species the venom apparatus is important also for the capture of food; among the coelenterates (corals and jelly fishes) stinging organs arranged in batteries serve this purpose; in the myriapods and spiders the venom glands are related to the mouth parts which provide an efficient mechanism for inoculation and a well-developed apparatus for injecting their poison is developed in the hinder parts of scorpions and wasps. The venom apparatus reaches its highest development in the poisonous snakes, in which the venom glands are specialized salivary glands and the venom is inoculated by canalised or grooved teeth; these venoms not only immobilise prey but aid in its subsequent digestion. Apart from the development of poisons with these functions the eggs and tissues of a number of species are poisonous, if administered parenterally. Finally, some fishes, crustaceans and molluscs, taken as food may cause serious effects because of the presence in them of poisonous substances which can be absorbed from the alimentary tract.

In the short space here available it is not possible systematically to survey all the recent work upon every species which elaborates poison. I shall therefore confine myself to a discussion of the nature and pharmacological action of those poisons whose chemistry has been most studied. The whole literature up till 1922 has been excellently reviewed by Marie Phisalix (1) and Gessner (2) has contributed a short but complete survey of advances up to the end of 1937.

## THE VENOMS OF THE AMPHIBIANS

The secretion of the "parotid" and other skin glands of toads (*Bufo*nidae) has been thoroughly studied, particularly by Wieland and his coworkers (3 to 6), by Jensen, Chen and Chen (7 to 10) and by Tschesche & Offe (11). A full bibliography is given by Gessner (2). The secretion contains substances which resemble in their pharmacological actions, and to some extent in their structure, the glucosides of digitalis and squill—*e.g.*, the bufotoxins, bufagins and bufotalins; basic substances, mostly possessing pressor action, are also present—*e.g.*, bufotenine, bufotenidine, bufothionine and epinephrine. Cholesterol, sometimes mixed with ergosterol (7), may also be present as well as vitamin C and glutathione (12) and certain unknown constituents which are responsible for the characteristic smell and for the local and haemolytic actions of the secretion (2).

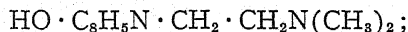
All the *Bufo*nidae which have been investigated have yielded digitalis-like substances, generally similar in action, but exhibiting specific differences in chemical structure. These substances seem to be related to the sterols, bile acids and sex hormones (4, 13). They probably possess a cyclo-pentenophenanthrene ring structure as was shown for cinobufagin by dehydrogenation with selenium (11, 14), and for pseudobufagin by Ikawa (15). Bufotoxins and bufagins (bufogenins) have been isolated from the crude venoms. The bufagins correspond to the aglycones of the cardiac glycosides and the bufotoxins may be regarded as combinations of the corresponding bufagins with arginine and suberic acid. Most of the bufagins have 24 carbon atoms or are acetyl derivatives of genins with 24 carbon atoms. For example, cinobufagin from *Bufo gargarizans* has the formula  $C_{24}H_{32}O_5(C_2H_2O)$ . Arenobufagin and regularobufagin have been assigned the formula  $C_{23}H_{32}O_5(C_2H_2O)$  indicating genins with 23 carbon atoms. All the bufagins are lactones with two or three double bonds, two of which are in the lactone side chain. The pharmacological activity of these compounds appears to be intimately related to the possession of a lactone ring (16). The occurrence of bufotoxins and bufagins together in the poisonous secretions has suggested to Tschesche (13) the possibility that enzymes are present in the animal which convert one to the other.

Bufotalin (17), isolated in crystalline form from the skin of *Bufo bufo bufo*, has been shown to be a monoacetyl derivative of an unsaturated trihydroxy lactone with 24 carbon atoms ( $C_{24}H_{34}O_5$ ). Chen, Jensen & Chen (7) failed to isolate this substance from the skin

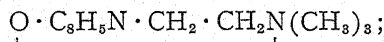
secretion of the common European toad and there is some doubt as to whether it is a constituent of the venom. A compound identical with bufotalin has been isolated from *Bufo formosus* by Kotake (18) and a somewhat similar pseudobufotalin from the Chinese drug Ch'an su by Kondo & Ikawa (19). Bufotalin is closely related to scillaren-A, having a similar lactone side-chain structure and almost the same ultraviolet absorption curve (5).

The activity of these compounds has been compared by Chen & Chen (16) with that of the cardiac glycosides. The cat units of nearly all the various bufagins are almost the same as, or about twice that of ouabain (0.1 mg. per kg.), those of the bufotoxins are generally two, three, or more times greater. These components of toad venom produce their effects upon the animal's own heart, are present in its circulating blood and indeed are essential to its proper functioning (20). They possess also the other toxic properties of the digitalis glycosides, causing nausea, vomiting, rise of blood pressure and contraction of smooth muscle. They are however eliminated more readily. The bufagins differ from the bufotoxins in exhibiting some local anaesthetic action. Except in regard to the production of emesis the suberylarginine part of the bufotoxin molecule does not appear to contribute to its toxicity.

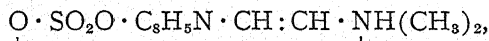
The specific basic pressor principles of toad venoms are tryptamine derivatives—a hypothesis put forward on pharmacological as well as chemical grounds by Jensen & Chen (21) and confirmed by Wieland, Konz & Mittasch (3). Bufotenin from *Bufo bufo bufo* is 5 hydroxyindolethyldimethylamine,



bufotenidine is the methylbetaine of bufotenine,



bufothionine, according to Wieland & Wieland (6), has the formula



being regarded as an internal ester of phenylsulphuric acid with the strongly basic dimethylamino group. They think it probable that bufogenin is formed in the cell from bufothionine by hydration. A number of bufotenines have been isolated from various species of

toads, chiefly by Chen, Jensen & Chen (7) and by Chen & Chen (8, 16). Some of these are common to more than one species.

Epinephrine has been found in the skin secretion of a number of toads, chiefly in those of tropical and subtropical distribution. It is not present in the European species. The pharmacological behaviour of crude toad venoms depends upon its presence. If present, its activity may overshadow that of the digitalis-like components, but if absent, the latter dominate the picture since the other active substances occur only in small amounts (2).

The secretions of the skin glands of many species of frogs are poisonous, though but few of them have been fully investigated chemically. Some behave like saponins causing haemolysis and contraction of skeletal and smooth muscle, both of which actions are antagonised by cholesterol. Others have strong neurotoxic as well as haemolytic actions. The secretions of some frogs contain pressor substances, for example that of the Australian *Hyla aurea* studied by Osborne (22). Gunn (23) found that the skin secretions of the South African *Xenopus laevis* had a powerful sympathomimetic action on the heart and vessels and on smooth muscle. Jensen (24) isolated from the secretion of this frog cholesterol and a base identical with bufotenidin.

Gessner & Craemer (25) extracted from the "parotid" and other skin glands of nearly a thousand salamanders 295 gm. of poison from which they obtained about 12 gm. of crude alkaloid base and 5 gm. of alkaloid salts. Schöpf & Braun (26) have isolated from this material an alkaloid, samandarine,  $C_{19}H_{31}O_2N$ . Its structural formula has not yet been determined but it probably contains 5 rings of which two are heterocyclic (one of them closed by the N of an NH group and one by an ether oxygen) and three are carbocyclic. This ring system carries at least two, and possibly three, methyl groups or side chains ending in methyl groups. The second oxygen atom is part of an hydroxyl group. The alkaloid is oxidised to a ketone, samandarone,  $C_{19}H_{29}O_2N$ , by heating with a mixture of chromic and sulphuric acids. Samandarine is a convulsive poison with close resemblances to strychnine and to picrotoxin, both of which it potentiates. It causes restlessness, increased reflex irritability, salivation, accelerated respiration, clonic convulsions, tonic spasms and finally central failure of respiration (27). It is a powerful analeptic, acting on the bulbar centres and causing increase in rate and depth of respiration and stimulation of the vasomotor centres. These effects are only of short duration (28). Samandarone has a similar action but only about half

the potency of samandarine. Other derivatives prepared by Schöpf & Braun were much less active (29). Samandarine has a transient local anaesthetic effect. It constricts the blood vessels in the frog but has a vasodilator action in mammalian preparations. It causes hyperglycaemia which is not determined by direct action of the alkaloid upon the suprarenals (30). The poison of salamanders also contains a cholesterol-like substance and unidentified active principles which give rise to the characteristic odour, to the strong local action, and to agglutination and haemolysis.

#### SNAKE VENOMS

Snake venoms contain at least two, and in some cases possibly more, toxic principles which are protein or of protein nature, some being enzymes. The classification of the toxic actions of venoms and the relation of these to separate active principles is still uncertain. Houssay's classification (31) may now be simplified and most of the toxic actions of venoms may be attributed to (a) proteolytic enzymes, (b) phosphatidases, and (c) neurotoxins. For one venom, that of *Crotalus terrificus*, the neurotoxin and phosphatidase actions are accounted for by a single protein constituent (32).

To proteolytic enzymes must be attributed the severe haemorrhagic and destructive effects at the sites of the bites of viperine snakes, some of the haemorrhagic effects in the viscera (31) and the coagulant properties of venoms. Eagle (33) found that venoms with coagulant properties fell into two groups. Those of the first, including some of the most powerfully proteolytic venoms, can coagulate pure fibrinogen *in vitro* without calcium ions, tissue extracts, platelets or prothrombin. The optimum pH is 6.5, as for thrombin, but coagulation is unhindered by specific antithrombin. The proteolytic enzyme in these venoms behaves like papain, which converts fibrinogen into a fibrillar gel. The venoms of the second group are unable to coagulate pure fibrinogen. They convert prothrombin to thrombin, and the optimal yield is generally the same as that obtained with tissue extract and calcium ions. The thrombin formed can be neutralized by anti-thrombin. The presence of tissue extract or of cephalin neither increases the rate of formation of thrombin nor its final amount. This type of activity is analogous to that of trypsin. Some of the crude venoms are many times more potent than pure trypsin. The non-coagulant venoms destroy prothrombin and, some of the more powerfully proteolytic, destroy fibrinogen also.

The proteolytic enzymes may contribute to the hypotensive action of snake venom by damage to vascular endothelium with escape of blood from circulation and possibly also by the liberation of histamine.

The phosphatidases also play an important part in poisoning by snake venom, causing haemolysis, and most of the effects on the heart and circulation. It is by no means certain that part or all of the neurotoxic effects of some venoms may not ultimately be found to be attributable to the phosphatidases.

The acceleration of venom haemolysis by lecithin takes place by the splitting off of one molecule of oleic acid from lecithin to form lysolecithin (34). This substance is actively haemolytic. It has effects upon the heart similar to those caused by venom. It acts upon isolated striated muscle *in vitro* and causes, like some venoms, contractures, fibrillation, increased imbibition of water, increase in lactic acid content, loss of potassium and phosphates, and inexcitability (35, 31). It attacks the capillary endothelium, causing haemorrhagic oedema of the lungs when injected intravenously, and oedema and necrosis when injected subcutaneously (36). Moderately large doses are required to kill animals by intravenous injection, probably because the proteins of the plasma afford some protection. Egg albumin in solution inhibits the haemolytic action of lysolecithin (37). It is of interest to note that the venom of wasps carries the hydrolysis of lecithin a stage further and splits off a second fatty acid, and may even liberate the choline and glycerol (37). Hughes (38) has observed the changes induced by venoms in the surface potential of a unimolecular film of lecithin. The optimum pH was 7.3; compression of the molecules in the film decreased the rate of hydrolysis. The haemolytic activity of Australian venoms was directly related to their lecithinase content. The lecithinase was stable to prolonged boiling at pH 5.9, but was rapidly destroyed in alkaline solutions. Cobra venom was more active in dilute than strong solution and was inhibitory in high concentrations, because coagulable protein in the venom is preferentially adsorbed on the film, forming a protective layer. Prehaemolytic swelling of corpuscles may be explained by the formation of lysolecithin which gives an expanded, fragile, and more permeable film, because the area per hydrocarbon chain is nearly double that in a lecithin film in the same state of compression.

The abrupt fall of systemic blood pressure which results when non-coagulant venoms are injected intravenously results in a large part from the action of phosphatidase by the formation of lysolecithin

and the liberation of histamine. Arthus (39) and his pupils studied this hypotensive effect and noted its resemblance to that occurring in acute anaphylaxis in the rabbit and dog. Essex & Markowitz (40) stressed the resemblance between the circulatory effects of *Crotalus atrox* and those in acute anaphylaxis and histamine poisoning and Kellaway & LeMessurier (41) explored the possibility that the circulatory action of the venom of the Australian copperhead (*Denisonia superba*) was caused by the liberation of histamine. Gautrelet, Halpern & Corteggiani (42, 43) showed that the hypotensive action of the venom of *Vipera aspis* was accounted for by peripheral vasodilatation and increased capillary permeability with increase in the number of red corpuscles and in the viscosity of the circulating blood, and that of the Indian cobra in the rabbit and dog was mainly a histamine-like effect on the capillaries.

Feldberg & Kellaway (44) found that the injection of the venom of *Crotalus atrox*, of *Denisonia superba* or of the Indian cobra (*Naia naia*) into the pulmonary artery of the perfused guinea-pig's lung caused the appearance of histamine and coagulable protein in the venous perfusate. The last two venoms when injected into the perfused lung of the dog also caused the liberation of histamine and coagulable protein and, when cobra venom was injected intraportally into the perfused liver of the dog, coagulable protein, pigments and histamine appeared in the fluid flowing out from the hepatic veins (45). Further analysis of the effects of intravenous injection of cobra venom in cats and dogs (46) showed that most of them could be explained by the liberation of histamine. Recovery from the initial precipitous fall of systemic blood pressure, if the dose of venom was not too large, was followed by a secondary fall of pressure leading to death. In the cat the immediate fall was accounted for by obstruction in the pulmonary circulation and the late fall by fluid loss from the circulation, peripheral vasodilatation, haemorrhagic oedema of the lungs and in some cases by failure of the heart. In the dog the initial fall of systemic blood pressure was shown to be dependent upon peripheral vasodilatation, with fluid loss from circulation, constriction of the hepatic veins and injury to the liver cells being contributory causes. Haemorrhagic congestion of the mucosa of the duodenum and of the upper part of the jejunum, like that caused by histamine, was also observed. Histamine exerts much of its effect at the site of its liberation. Dragstedt, Mead & Eyer (47) have apparently overlooked this in concluding, from experiments in which they demon-



strated only small amounts of histamine in the blood and lymph of dogs after the injection of *Crotalus* venom, that liberation of histamine plays merely a subsidiary part in the production of symptoms by this venom.

The study of the mechanism of the liberation of histamine by snake venoms has led to the recognition of the rôle of phosphatidase. Lysolecithin is formed in perfused organs when snake venom is injected and itself liberates histamine from perfused organs. Its formation is an intermediate step in the liberation of histamine by venoms (45). There is also formed a substance which causes slow and delayed contraction of the isolated jejunum of the guinea pig and subsequent changes in the reactivity of the muscle to histamine and acetylcholine. The same substance is formed, together with lysolecithin, when snake venom acts upon egg yolk *in vitro*. The contraction of smooth muscle by venoms is accounted for in part by the liberation of histamine in the muscle and in part by the formation of this substance.

The part played by lysolecithin in snake-venom haemolysis is parallel to its action in liberating histamine. Trethewie (48) has compared these actions in the case of two Australian snake venoms, that of the black snake (*Pseudechis porphyriacus*), which is strongly haemolytic and that of the death adder (*Acanthophis antarcticus*), which is only feebly haemolytic. The latter venom needs to be present in higher concentration than the former to cause a large output of histamine. A further study of lysolecithin (49) has stressed the close similarity between many of its actions and those of snake venoms.

The "neurotoxins" of snake venoms possess various actions on the nervous tissues. Houssay & Hug (50), using the method of crossed circulation of the isolated head, showed that the venom of *Crotalus terrificus* has a direct action on the bulbar centres and Gautrelet, Halpern & Corteggiani (42, 43) demonstrated that the venom of *Vipera aspis* and that of the Indian cobra (*Naia naia*) have no such action. Very few venoms have been studied by this method and the evidence of a less direct kind for central action offered, for example, in the case of the venom of the Indian cobra by Chopra & Iswariah (51) is less convincing.

The curari-like action of many snake venoms upon motor-endings [for literature see Kellaway (52)] and the paralysing action upon sensory endings (53) may be caused by a "neurotoxic" factor or may even be accounted for by the action of phosphatidase, though quantitative considerations make this last possibility unlikely.

Apart from these three groups of actions certain other enzymic actions of venoms should be noted. Chain (54) followed up E. Mellanby's observation that cobra venom caused a decrease in the uptake of oxygen and in glycolysis by cancerous tissue slices. He showed that certain venoms contain a thermostable substance which inhibits glycolysis and alcoholic fermentation. This anti-fermenting principle is neutralised by specific antivenene. Ghosh (55) and Ghosh & De (56) found that the proteases of the venoms of the Indian cobra and Russell's viper resemble trypsin in their optimal pH. The venoms also contain a peptidase with similar optimal activity to erepsin, a trypsin inhibitor and an enzyme which converts haemoglobin to methaemoglobin. Gulland & Jackson (57) found phosphomonoesterase and phosphodiesterase in a number of venoms and phosphodiesterase alone in others. They found 5-nucleotidase occurring richly in the venoms of Russell's viper, the water moccasin, the diamond rattlesnake and the banded krait. It dephosphorylated specifically adenosine-5-phosphate and inosine-5-phosphate but failed to attack a number of other monophosphate esters.

Much progress has been made in the isolation of various active principles from snake venoms but we cannot yet be certain whether the venoms are complexes of proteins as suggested by Slotta & Szyska (59) or mixtures of separate active constituents. Possibly several enzyme actions may be related to a series of active groups in a single protein molecule. In any case we cannot assume that all the substances which have been isolated are actually unaltered in the process, or that the results obtained with one venom can be transferred to another. Apparently conflicting results achieved by different workers with venoms even from closely related snakes need not lead to the conclusion that one or the other is certainly in error.

Dunn (60) precipitated the coagulable protein of the venom of *Crotalus adamanteus* by heat and obtained an active cephalinase from the filtrate by precipitation with 95 per cent alcohol. This fraction was five times as potent in haemolytic action as the original venom. He obtained a proteolytic fraction by selectively adsorbing cephalinase with aluminium hydroxide C. The shock-producing factor was not removed by precipitation of heat-coagulable protein and was not identical with cephalinase, for it was soluble in alcohol. Dunn removed from this venom by adsorption another active principle, that which converts haemoglobin into methaemoglobin.

Ghosh & De (61) by combining adsorption and elution with frac-

tional precipitation have concentrated the "neurotoxin" of cobra venom in a protein fraction containing 21.3 per cent of the protein of crude venom. Micheel & Jung (62), by ultrafiltration (which purified and concentrated the active constituents), dialysis through cellophane (which allowed the active principle to pass), and concentration *in vacuo*, obtained from the venom of the African cobra, *Naia flava*, a neurotoxin which on analysis was found to contain carbon 45.2 per cent, hydrogen 7 per cent, nitrogen 14.7 per cent, sulphur 5.5 per cent, and ash 3 per cent. The molecular weight of this protein-like substance was estimated to be between 2500 and 4000 and it had a minimum lethal dose of 0.12  $\mu$ g. per gram of white mice. A second constituent of smaller molecular size with a minimum lethal dose of 1  $\mu$ g. per gram was also obtained. Micheel & Bosser (63) were not successful in applying the same methods to the venoms of a number of New World species including those of *Crotalus terrificus*, *Bothrops jararaca* and *Agkistrodon piscivorus*. The neurotoxins of these venoms are of larger molecular size and do not dialyse through cellophane. The poison of *Agkistrodon* was peculiar, since when dialysed the venom lost half its activity, some of which was regained by reuniting it with dialysate. The venom apparently contains a dialysable activator. Micheel, Dietrich & Bischoff (64) used ultrafiltration, dialysis, fractional precipitation and kataphoresis with a high-voltage current (3000 to 3500v) through a series of cells, the venom or fractions being dissolved in conductivity water and a very small current passing (2–20 ma.). The fractions tended to accumulate in cells at a pH near their isoelectric point. From *Naia flava*, in addition to neurotoxic protein A, (62) they obtained a fraction B with a minimum lethal dose of about 1  $\mu$ g. per gram. From *Naia naia* they obtained a neurotoxin A with a minimum lethal dose of 0.08  $\mu$ g. per gram (in another experiment an unstable fraction with a minimum lethal dose of 0.03  $\mu$ g. per gram), and also a crystalline neurotoxin C containing zinc. It had a minimum lethal dose of 6–9  $\mu$ g. per gram of mouse (by analysis, zinc 22.37, carbon 25.7, hydrogen 3.83, nitrogen 6.04 per cent). The neurotoxins could be oxidised by shaking with cuprous oxide in alkaline solution and the process was reversed with cysteine or glutathione in acid solution. The authors develop the hypothesis (62) that the active fractions contain a thiolactone ring. The activity is removed by bisulphite and the reaction is attended by the appearance of sulphydryl groups, change in optical rotation and the formation of a precipitate with a sulphur content higher than

that of the original venom. The active fractions of protein nature are more unstable to heat than is the whole venom. Slotta & Fraenkel-Conrat (65) found that the venom of *Crotalus terrificus* had a high sulphur content but contained no free sulphydryl groups, the sulphur being present in disulphide form. Cysteine acted on the venom breaking the disulphide bonds ( $—SS—$ ) with loss of activity of the neurotoxin. They doubt the reversal of inactivation of neurotoxin of cobra venom by cysteine in acid solution and suggest that the results of Micheel and his colleagues may be explained without the assumption of a thiolactone ring, by the cleavage of disulphide bridges to yield a thiol and an unstable thio sulphonc acid. Micheel & Schmitz (66) later found that the purified neurotoxins of cobra venom are weakened by a large excess of cysteine only to a slight extent, and that this is because they still contain about 25 per cent of a component which is inactivated by cysteine. This inactivation is irreversible. Micheel & Bode (67) found that inactivation of cobra neurotoxin by sulphite probably does not take place by the formation of thiol groups. They appear to have excluded the possibility of the presence of thiolactone or of disulphide groups in the active portion of the neurotoxin. Inactivation with sulphite takes place with increasing velocity as the pH rises, whereas these groups are split by sulphite more quickly with decreasing pH. An easily dialysable substance is formed during the inactivation of the neurotoxin. This they obtained as a crystalline sodium salt with the formula  $C_7H_{14}O_{11}N_2(SO_3Na)(COONa)$ .

Klobusitzky (68) has obtained from the venom of *Bothrops jararaca* a nitrogen-free neurotoxic principle, four-and-one-half times as active as the original venom but devoid of haemolytic activity (cf. 32); it had about one-fifth the coagulant activity of the whole venom. Klobusitzky & König (69) have since separated the coagulant enzyme and obtained it in solution free from neurotoxin.

Tetsch & Wolff (70) treated the venom of *Crotalus terrificus* with watery picric acid and obtained from the resulting precipitate, by further treatment with acetone, a snow-white powder of protein nature (by analysis, carbon 44.9 per cent, hydrogen 6.6 per cent, nitrogen 13.7 per cent, and sulphur 3.6 per cent) soluble in water and in ethyl and methyl alcohol.

Slotta & Fraenkel-Conrat (32) report the isolation of two active proteins from the venom of *Crotalus terrificus*. About 10 per cent of the venom is an albumin-like blood coagulating principle which is proteolytic; this they were able to concentrate tenfold by precipita-

tion with 40 per cent ammonium sulphate. The inactive globulin precipitate had the active substance adsorbed upon it. When the ammonium sulphate was removed by dialysis the activity remained in the supernatant liquid from which the globulins separated out. Further concentration was achieved by evaporating the liquid after freezing *in vacuo*. The neurotoxic principle, crotoxin (which is about 60 per cent of the whole venom) has been crystallized and contains the whole of the neurotoxic and haemolytic activities of the venom. It was isolated by heat coagulation, precipitation at the isoelectric point and ammonium sulphate fractionation. It crystallizes from solution in pyridine acetate in thin quadratic plates; it contains 4 per cent sulphur, at least partly in disulphide form. Reduction with cysteine inactivates the toxin. A minimum molecular weight of 11,000 has been calculated from the methionine value. The molecular proportion of methionine to cystine is 1:6 and it is suggested that the molecular weight of the protein is either 3 or 6 times 11,000. Gralén & Svedberg (71) have since found that crotoxin behaved in sedimentation and diffusion as a homogeneous substance and calculated its molecular weight as 30,000. The molecule was split into smaller inactive substances by warming in alkaline solution or by treatment with cysteine.

Slotta & Forster (72) found that not all of the sulphur in crotoxin can be accounted for by cystine and methionine and suggest that the remaining sulphur is present in some form other than the commonly recognized disulphide, sulphydryl, thiolactone or thiol. They found that the purified active principle of *B. jararaca* also contains cystine, 5.74 per cent, and methionine, 1.08 per cent.

#### BEE VENOM

Bee venom resembles snake venom very closely, it has phosphatidase (haemolytic) and neurotoxic properties. It produces histamine-like effects (73) and contains histamine (70, 74). Even when this is removed, it produces in the cat and dog effects which are closely similar to those of cobra venom. It causes the liberation of histamine from the perfused lungs of the guinea pig and dog and from the perfused liver of the dog (75).

Reinert (74) purified bee venom by extraction with water and precipitation with alcohol. The whole powder so obtained contained no sulphur and less than 0.3 per cent phosphorus (carbon 45.7 to 47.4 per cent, hydrogen 7.3 to 7.8, nitrogen 13.8 to 14.5, ash 2.4 per cent). This proteose-like substance contained 8 per cent of trypto-

phane. It resembles a saponin in its haemolytic properties and in its detoxification with bromine or tannin. The haemolytic and neurotoxic activities of the venom are not combined in the same active principle, because the neurotoxic action is destroyed by acid hydrolysis and the venom so treated is still actively haemolytic. Tetsch & Wolff (70) obtained from bee venom collected in summer a sterol-like substance by extraction with ether and precipitated the neurotoxin with watery picric acid. The precipitate by treatment with acetone yielded a snow-white powder (carbon 43.6 per cent, hydrogen 7.1 per cent, nitrogen 13.6 per cent, sulphur 2.6 per cent). The minimum lethal dose for mice was 6 to 10  $\mu$ g. per gram. Hahn & Ostermayer (76) extracted the stings and poison sacs of eight thousand bees with formic acid and obtained 3.247 gm. of crude dry venom. This was purified by extraction with 60 per cent alcohol and gave 1.234 gm. of active substance, the residue being inactive. Treatment with ammonia in watery solution and final evaporation gave a protein containing nitrogen 14.2, sulphur 1.53, and phosphorus 0.44 per cent. Hahn & Leditschke (77) found that magnesium was present to the extent of 0.4 per cent in the crude venom and was removed by the treatment with ammonia as magnesium ammonium phosphate. Hahn & Leditschke (78) obtained further purification by dialysis and separated their neurotoxin into two components. One was a convulsive poison which was heat-labile and passed through the membrane. The other, which caused paralysis in mice, was practically non-dialysable, not inactivated in two hours at 100° C at pH 4, and was basic and insoluble in alkaline solutions. The two components gave almost identical ultraviolet absorption curves and each contained about 1 per cent of sulphur. The convulsive neurotoxin is possibly a phosphoric acid derivative of the paralytic component.

#### PARALYTIC SHELLFISH POISONING

The origin of shellfish poisoning in sporadic outbreaks in Europe has never been satisfactorily explained. The outbreaks since 1927 on the Pacific coast of North America have been studied by Meyer & Sommer and the paralytic poison has been extracted in very active form by Sommer (79). The appearance of toxicity in shellfish has been shown to be associated with their taking as food a dinoflagellate, *Gonyaulax canella*, from which the paralytic poison has also been obtained. Three other poisons from shellfish have been obtained, one of which is probably a quaternary ammonium salt; one which is much

less potent only manifests its activity after a characteristically long latency (80). The paralytic poison is absorbed from the gastrointestinal tract and rapidly eliminated by the kidneys; it has a depressant effect on blood pressure and respiration and impairs conduction in the heart (81). It is a powerful neurotoxic poison with central and peripheral actions, is strongly curarizing and paralyzes sensory endings in minute concentration (82).

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## RUMINANT NUTRITION\*

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In this brief review an attempt has been made to comply with the Editors' invitation to critically appraise the present status of the nutrition of ruminants and to concentrate on those topics in which significant progress is being made. Since this is the first review on the subject in this series, it is deemed advisable to extend the discussion to the literature appearing over a number of years so as to render the matter more intelligible. Owing to the breadth of the field and the exigencies of space, consideration of many important contributions has been omitted, and several subjects have been set aside altogether for future review.

Research concerning ruminant nutrition has been for the most part inspired by problems affecting the immense animal industry which supplies civilized man with meat, milk, and wool. The variety of conditions under which animal production is conducted in individual countries has stimulated entirely different lines of investigation; those of the old world are devoted mainly to intensive feeding practice, while those of recently settled areas are concerned more especially with nutritional disabilities imposed on the grazing animal by climate and terrain.

### ENERGY METABOLISM

Interpretation of the older literature devoted to the metabolic rate of fasting ruminants is complicated, because the prerequisites of the determination have not always been fully realized. In standardising the physiological conditions to make measurements comparable, it is now generally understood that the animal must be subjected to an environmental temperature in the zone of its thermic neutrality, the precise limits of which are imposed by the amount of insulation afforded by the skin cover; that emotion and gross activity must be absent, the animal preferably should be in repose; and that the processes of digestion and absorption of food must be no longer in operation, the animal should be in a post absorptive state with a respiratory quotient approximating that of fat, 0.73.

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When these conditions are strictly adhered to and all possible sources of error in the estimation are overcome, the standard fasting metabolism of ruminants has been observed to be extraordinarily variable. Lines & Peirce (49) found from a critical study of the standard metabolism of Australian merino sheep, that after fasting for forty-eight hours, ewes off dry autumn pastures, poor in protein but providing sufficient nutriment for maintenance, had a metabolic rate of 1060 kilocal. per sq.m. per day. After grazing on lush green pastures for a period prior to the determination, the average rate was 1250 kilocal. per sq.m. per day. Each set of data was referred to the previously determined area-weight relationship ( $A = 0.091 W^{2/3}$ ). In these studies, it was established that animals subjected to an unvarying dietary regime have a remarkably constant fasting metabolism, and that the metabolic rate, even after three days' fast, is materially affected by the previous plane of nutrition. The respiratory quotients of these subjects, under the conditions of the determinations, fell into a group with a mode of 0.73 and a mean of 0.72. In a later investigation Peirce (89) observed a variation of 30 per cent in the forty-eight hour fasting metabolism of grazing ewes. This reached the high level of 1350 kilocal. per sq.m. per day off spring pastures and receded to 1040 kilocal. per sq.m. per day in late autumn when grazing conditions were poor.

Benedict & Ritzman (7, 8) in a contemporary study of Rambouillet-Southdown sheep in New Hampshire observed similar differences, and stressed also the influence of previous nutritional history on the fasting metabolism of sheep. The mean of their data was approximately 13 per cent above that obtained in this laboratory. Benedict (5) believes that racial difference between the two groups of animals might explain the discrepancy, since variations of a larger order between different human races have been described. The effects of dietary regime and the emotional state of the experimental animals may, however, have contributed materially to this difference.

The fasting metabolic rate of young lambs is considerably greater than that of mature sheep (89, 7, 8). The insulating effect of the dense fleece has no evident effect on the standard metabolic rate, since the energy exchange of fasting merino ewes ten days prior and twenty days subsequent to shearing has been observed to be unchanged (89).

The heat production of the sheep is greater in the standing position than in normal resting posture, because of the muscular effort involved. Peirce (89) considers this increase is approximately 8 per

cent, Hall & Brody (28) from 7 to 10 per cent, and Ritzman's observations (8) indicate that the difference is 15 per cent. Benedict (5) suggests a correction of 10 per cent as a compromise, when comparing the standard metabolic rates of standing and reclining sheep. Studies of Ritzman, Calovos & Benedict on the effect of castration (94) were complicated by lack of control of the dietary regime previous to the determination of the fasting metabolism. They concluded that castration of the male at puberty or in adolescence results in a 5 to 10 per cent decrease in the call for energy, and they ascribe the major part of this effect to the absence of testicular hormone.

The fasting metabolism of the goat is very materially less than that of sheep of similar weight. Ritzman, Washburn & Benedict (95) observed that does with a mean body weight of 36 kg. dissipated on an average 680 kilocal. per  $10 W^{2/3}$  per day, which suggests that ewes of similar weight have a standard metabolism which is 65 per cent higher.

Beef cattle, and more especially dairy cows, evidence a very labile metabolism. Ritzman & Benedict (93, 6) have reported fluctuations up to 80 per cent in the standard fasting metabolism of cows which were neither pregnant nor lactating. These observations were carried out usually on the fourth day of fast.

The considerable variation and extreme lability of the fasting metabolism of the three ruminants that have been carefully studied, lend little support to some of the attempts that have been made to establish a comprehensive unifying principle relating inter- and intraspecific basal metabolic rates. But while mathematical treatment of the observations in the literature has at times exceeded the bounds of reason, the general indication is that the logarithm of the basal metabolism is approximately proportional to the logarithm of the body weight of most homeotherms. Ruminants, however, deviate considerably from this relationship. Benedict (5) has recently discussed the evidence.

As the metabolic rate obviously does not vary directly with the body weight, a great deal of effort has been expended during the last century in an attempt to establish some basis to which the metabolism of animals of different weight might be referred. The history of the subject has been generously treated by Harris & Benedict (30) and more recently reviewed by Kleiber (41).

Rubner's belief that the temperature difference between the homeotherm and its environment controlled the heat loss and so conditioned its metabolic rate, may be set aside as neither the findings

nor theoretical considerations based on Fourier's law of energy dissipation from surfaces lends support to the contention that basal metabolism bears a closer relationship to the geometric surface of animals than to some other function of their body weight. Experimental findings that metabolism varies exponentially with weight are, however, independent of any theoretical explanations of causal relationships which have given rise to a half a century of polemics.

The literature on basal metabolism is difficult to interpret on account of the many factors that have been utilized by different authors. The search for a common basis of comparison between animals of different sizes and of different species has led to the reasonable compromise summed up in the conclusions (91) of the Committee of Animal Nutrition of the National Research Council (U.S.A.), who recommend . . . "that the factor, body weight raised to the 0.73 power, be temporarily adopted as a base of reference for computing fasting heat production and endogenous metabolism."

Since Thayer's classical attempt to evaluate foodstuffs empirically in terms of hay, different means of approach have resulted in the several feeding standards for ruminants in current use.

The hay system, modified by Fjord and sponsored by Hansson (29) finds its expression in the Scandinavian feed unit; Grouven's early suggestions, extended and elaborated to include only so-called "digestible nutrients," still forms the main basis of widely used American tables (32); while the Kellner-Armsby net energy principle in different guises finds extensive support in Central Europe and the British Isles.

Exigencies of space do not allow a discussion of the relative merits of these guides to feeding practice. Whether the capacity of various fodders to fulfill the energy requirements of ruminants is best expressed in calories of digestible nutrients, of metabolisable energy, or of net energy may not be decided until the true physiological significance of each of these terms is defined (93). The logical principle of estimating the nutritive value of a fodder by its ultimate usefulness to the animal rather than from its chemical composition is, however, generally conceded, but in spite of the greater scientific accuracy implied in the net-energy concept,<sup>1</sup> feeding standards based on it are

<sup>1</sup> The total combustible energy of the feed minus the energy expenses of utilization yields the net energy available for maintenance and production (24). These expenses are the potential energy of the visible excreta and of the methane arising from bacterial cleavage of carbohydrates, together with the heat in-

by no means beyond reproach. The theoretical superiority of the energy-balance method is unassailed, but the demands of feeding practice for generalisations have at times resulted in misleading information arising from attempts to assess fodder values by means of net energy determinations which have failed to appreciate certain essential factors.

Consideration of the data arising from extension of the studies initiated by Armsby, led Forbes and his collaborators (24) to doubt the assumption that heat production is a rectilinear function of the quantity of feed, and this in turn led to the splendid series of experiments planned to investigate the relationship which energy metabolism bears to the plane of nutrition. A study (23, 24) of the complete energy balances of four steers fed at several levels ranging from fasting through submaintenance to a high production level of three times maintenance, demonstrated that the curve relating plane of nutrition to energy expenditure was sigmoid in shape; the observed heat increment increased slowly between fasting and maintenance, rose rapidly above maintenance, and fell off between the highest planes of nutrition where it would seem the maximum stimulus had been exceeded. The authors concluded that the most practicable possibility of deriving a system of energy values of feeding stuffs requires continued adherence to the general point of view expressed by Armsby, but modified in the light of more recent experience by adoption of the heat production of fasting under standardised conditions<sup>2</sup> as a base value in the determination of energy metabolism and as a measure of the maintenance requirement of net energy. Recognition must also be given to the different rate of the economy of utilization of food energy for body increase and production as compared with maintenance.

Mitchell and his collaborators (78), from observations obtained under somewhat similar conditions to those of Forbes, conclude also that the net energy of a ration cannot be assigned any one value. Mitchell suggests, however, that it may be predicted for all practical

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crement resulting partly from the direct expenditures of energy in the mechanical processes of prehension, mastication, deglutition, rumination, and peristalsis, and partly from the chemical energy expended in digestion, anabolism, excretion, and the chemical stimulus to metabolism (calorigenic action).

<sup>2</sup> The heat production of the first day during fasting following upon the appearance of a metabolic state characterized by the non-protein respiratory quotient of fat; this measurement to follow a preparatory period of feeding at the plane of energy equilibrium.

levels above maintenance from a linear relationship. Below maintenance, this linear function does not hold.

In view of this, it may no longer be assumed that the efficiency of utilization of energy is the same above the maintenance level as it is below, and so the existence of fundamentally different net energy values of a fodder at different planes of nutrition becomes apparent. The interpretation of existing tables of the relative capacity of different fodders to supply productive energy thus calls for careful consideration. In general, it may be assumed that such of Armsby's net energy values as are based on Kellner & Kohler's observations represent more closely actual production values, while those based on the experiments of Armsby & Fries, especially the values for roughages, have been invalidated. Since Møllgaard utilized basal rations sufficient to cover maintenance requirements, his determinations of net energy for production are seemingly not vitiated, other than insofar as they imply the existence of a constant relationship between net energy values of a food for the different functions of maintenance, of body increase and of milk production (23).

Wiegner & Ghoneim (118) have attempted a quantitative generalisation of energy utilization by postulating that successive increments of net energy decline with successive increase of food consumption in accordance with the expression  $\frac{dA}{dF} = K (H-A)$ , in which  $A$  is the net energy,  $F$  the metabolisable energy,  $H$  the maximum net energy of food intake and  $K$  the efficiency coefficient. The integrated form accounts with reasonable accuracy for the data from the steers of Forbes *et al.* and for their own experiments with rabbits.

The ruminant differs from other herbivora in the degree of efficiency with which it derives energy from cellulose and similar complex carbohydrates. The utilization of "crude fibre" from plant materials is evidently limited by the extent of lignification since the digestibility of highly lignified straw may be approximately doubled by pretreatment with hot alkali (22, 33). The conversion of cellulose into materials which may be absorbed and utilized is practically all accomplished in the voluminous paunch and subsidiary organs which constitute the pre-stomachs (26). The fermentative process is exothermic (45) and is accompanied by the evolution of a considerable volume of methane. The claim that hydrogen in varying amounts accompanies the methane formed during rumination, has been seriously questioned (44, 50), and it would appear that methane and car-

bon dioxide are the only gaseous products. Woodman, who based his views on the behaviour of thermophilic cellulose splitters which in the presence of toluene produce reducing sugars in demonstrable amounts, believed that cellulose digestion in the ruminant proceeds through cellobiose to glucose (120). In light of the fact that reducing sugars do not appear in the actively digesting contents of the rumen, and in view of the large volume of evidence that the end products of cellulose digestion are fatty acids, he has recently reviewed the situation (121) and has abandoned his hypothesis which stated that the capacity of cellulose to supply useful energy to the ruminant could be attributed to glucose formation in the paunch.

Studies (67) of the course of degradation of cellulose by means of fermentations *in vitro* with mixed cultures removed from the rumen through fistulae, have indicated that the non-gaseous products are practically all volatile fatty acids and, furthermore, that with such mixed cultures as normally occur, the milieu of the rumen determines the nature of the final products. The physico-chemical environment of the rumen is seemingly extraordinarily constant. Observations on samples withdrawn from the rumen without loss of carbon dioxide indicate that the reaction varies but little from pH 7.2 (67). It would seem that the very considerable production of acid is regulated by the continuous flow of parotid saliva (97) which in the sheep provides from seven to ten liters (64) of approximately 0.2 *N* alkali bicarbonate (107). Colin, in his classical studies, has estimated that the parotid saliva secreted by a young ox is of the order of sixty liters per day, and contains approximately 300 gm. of alkali bicarbonate (58).

Studies of the effects of synthetic diets composed of cellulose, protein, salts, etc. (51) and observations *in vitro* (67) have rendered clear the exacting demands of the microflora of the rumen for various adjuvants which normally occur in the natural diet.

In order to reconcile with the above findings Kellner's claim that digestible cellulose and digestible starch provide the same amount of productive energy for ruminants, search has been made for possible sources of glucose formation during the digestion of cellulose. Pochon (90) considers that the alteration in reaction of the contents of the rumen when they pass to the more acid abomasum (true stomach) might arrest the cleavage of cellulose at a stage where glucose is produced. While this may occur, evidence from other studies suggests that it plays a minor role in the production of assimilable energy from



cellulose. Another possible source of glucose from cellulose is through the agency of the starch and glycogen stored by the infusoria which normally inhabit the rumen in immense numbers (56). This again seems not to be quantitatively significant.

The activity of the microorganisms which convert cellulose, at the cost of a proportion of its potential energy, into substances which are absorbed and utilized by the ruminant, gives rise to carbon dioxide and methane, and so the fermentative process must be taken into account in the study of the energy metabolism of ruminants. Krogh & Schmidt-Jensen (44) by means of a study *in vitro* of the gases evolved from material withdrawn from the paunch found that the  $[\text{CO}_2]/[\text{CH}_4]$  ratio in different experiments varied between 2.95 and 2.2. The milieu was not especially controlled in these studies and the rate of fermentation observed was very much less than that which normally obtains in the rumen.

In expressing the energy balance sheet of fed ruminants, Andersen (1) includes the heat production arising from fermentation by augmenting the oxygen consumed by the animal with the amount necessary to burn the methane which is produced, and by adding to the total carbon dioxide evolved by the animal that which would be formed by the combustion of the methane. From these totals, equivalent calories may be computed by the well-known method of Zuntz & Schumberg. This procedure is superior to others in current use, as it is thermodynamically valid.

If, as the existing experimental evidence implies, the ruminant derives practically all of its energy requirements from cellulose, and if the end products arising from bacterial cleavage of this material in the rumen are fatty acids, an extraordinary intermediary energy metabolism might be expected. Little or no reference is made in the literature to this interesting implication.

#### PROTEIN REQUIREMENTS

The complications which attend the formulation of energy requirements of ruminants are overshadowed by the confusion in the existing literature relating to protein requirements. These are almost universally quoted in terms of a given weight of "digestible" food protein per unit body weight and are a survival of the time when differences in the chemical constitution of proteins were unknown. The general situation has changed very little since Mitchell (76) critically appraised the literature some years ago. The difficulties involved in

compounding protein-deficient rations of which ruminants will consume sufficient to fulfil their energy, inorganic, and vitamin requirements are appreciable but not insurmountable.

The maintenance quota of protein (of 100 per cent biological value), as implied by the values for endogenous urinary nitrogen, seems to vary exponentially with the body weight. From a mathematical analysis of recorded determinations, Brody and his collaborators (12) suggest that the ratios, endogenous nitrogen/basal metabolism, and urinary neutral sulphur/basal metabolism, are constant.

As a result of Zuntz's suggestion (122) that ruminants may derive protein from non-protein nitrogen through the propagation of bacteria in the paunch, many attempts have been made to substitute part of the protein in the ration with urea, amides, and ammonium salts. While there is little doubt that the addition of certain simple compounds of nitrogen to some rations stimulates the bacterial cleavage of cellulose and so enhances the supply of available energy, the hypothesis that protein in useful amounts is synthesised through the agency of the micro-flora of the rumen is, on the whole, far from convincing. Völtz (113), who was the first to investigate this matter, claimed that one of his experimental lambs consumed for eight months a diet very low in protein, consisting of urea, starch, alkali-washed rye straw, and inorganic salts, and increased during this period 40 per cent in body weight. The diet was not supplemented with accessory food factors. Neither subsequent work of Völtz and his coworkers (114, 115) nor critical investigations in other Institutes (96, 34) has lent support to the contention that simple nitrogenous compounds may be converted to bacterial protein in sufficient amount to fulfil a material part of the protein requirements for growth of the ruminant. Scheunert and his collaborators (96) doubted the obvious interpretation of the apparent improvement of nitrogen balance which followed the addition of urea supplements to deficient rations. They sought for other paths of excretion and found that the balances had been vitiated by the secretion of much urea in the sweat. By washing their sheep sufficient nitrogen to account for the discrepancy was recovered. The older literature on this subject has been considered in detail by Mitchell (77). Recently, independent groups of workers (19, 40) have again claimed that ammonium salts may be substituted for the major part of the extra ration fed to milk-producing cows. The evidence is little more conclusive than that of previous investigators. The conventional feeding standards which have been used for assess-

ing the amount of protein necessary for maintenance and for milk production seem to provide far in excess of actual requirements, and the duration of the experiments is, for the most part, insufficient to eliminate the carry-over effect of the dietary regime previous to the periods of observation. Morris (80) has demonstrated that although the urinary nitrogen will fluctuate greatly, the milk yield may remain unchanged for sixteen days after decreasing the protein level of the feed.

### WOOL GROWTH

The selective breeding of ruminants for high and efficient production of wool has given rise to a number of special problems of nutrition. The contrast between the high cystine content of wool (59) and the reputedly low cystine content of most vegetable proteins led to the hypothesis that when the protein in the pastures is low, the amount of available cystine in the fodder might impose a limit to the production of wool fleece by grazing sheep (73). Marked improvement in wool growth has been observed (60, 61) to follow the provision of a supplement of good quality protein to sheep on protein-deficient pastures, and direct study of the rate of wool production of sheep on a fixed ration, proven to be limited in its capacity to supply the assemblage of amino acids necessary for maximum wool growth, has demonstrated that an additional supply of 1 gm. of cystine per day stimulates wool production. The observed increase was trebled by injecting the same amount of *L*-cysteine subcutaneously, so as to avoid destruction of the free amino acid by the bacteria of the rumen. Injection of an equivalent amount of *L*-methionine under identical circumstances was followed by an increase of wool growth which was barely significant (62). The recent unequivocal demonstration (119) that methionine may take the place of cystine in the diet, and the observation (14) that methionine may be converted into the cystine necessary for hair growth in the rat, suggests that the slow steady absorption subsequent to the conversion of injected cystine into sparingly soluble cystine in the mildly oxidizing subcutaneous tissues may have resulted in its more efficient utilization in the above experiment. Consideration of recent evidence should lead to the broadening of the hypothesis to embrace the two sulphur-containing amino acids, cystine and methionine. No other naturally occurring sulphur compound has been shown to be capable of conversion by the animal to cystine. Variation of the concentration of protein in a fixed ration has been observed to exert extraordinary influence on the quantity

and quality (fineness) of wool grown by high-producing merino sheep (65).

While reassemblage of the amino acids of the fodder into the proteins of milk, ordinarily does not seem to be subject to limiting factors of similar magnitude as are experienced in wool production, considerable difference between the quality of different proteins for milk production is recognised. Adherence to current "feeding standards" has obscured the results of some investigations into this aspect of ruminant nutrition.

#### INORGANIC DIETARY CONSTITUENTS

Mitchell & McClure (79) have recently catalogued and briefly discussed a large volume of literature dealing with the inorganic requirements of animals, and in this publication much of the work cogent to the inorganic metabolism of ruminants has been mentioned. The following brief outline covers several aspects which have not been especially dealt with.

*Phosphorus in ruminant nutrition.*—The association of certain maladies which occur in cattle with grazing conditions on soils of low phosphorus content, was recognised in the early 'eighties of last century by Hutcheon (35) who stressed (36) the fact that the perverted appetite and physical disabilities suffered by cattle on the deficient veldt in South Africa could be prevented by supplying them with additional phosphate. The extensive investigations conducted by Theiler and his associates (104) proved that the fatal *Lamsiekte* is primarily caused through the development of a specific osteophagia associated with phosphorus deficiency, which leads the animals to ingest carcass debris infected with the toxigenic saprophyte *Clostridium botulinum*, and that the pathological condition of the skeleton, *Styfsiekte*, has much in common with osteomalacia. Augmentation of the supply of phosphorus to grazing cattle in South Africa has not only controlled the incidence of botulism, but has undoubtedly increased production (105), and the beneficial effect which supervenes when cattle which graze on certain areas are provided with phosphorus-rich concentrates has since been observed by many investigators in other countries (18, 108, 51, 79).

Apart from the pathological changes in the skeleton which have been discussed by Theiler (103), the most striking effect of bovine aphosphorosis is the lack and perversion of appetite, which have been recorded by practically all observers. This symptom is seemingly the

result of changes in intermediary metabolism imposed by the decreased concentration of circulating phosphate, since the animal's capacity to digest phosphorus-deficient rations is unaffected (17, 64). Kleiber and his coworkers (42) have studied the effect of phosphorus deficiency on the ability of cattle to utilize energy, and conclude that, notwithstanding the absence of any marked effect on fasting metabolism, the partial efficiency of food utilization and the efficiency of food protein for sparing body protein are both decreased.

The effects of uncomplicated phosphorus deficiency, experimentally induced in sheep, have been studied by Martin & Peirce (74), who report upon the persistently low blood phosphate, the lack of appetite and consequent decrease of growth increment, and the anatomical, histological, and chemical changes in the skeleton similar to those of osteoporosis and osteomalacia previously described in cattle. The findings have been confirmed by Stewart (100) and the bone pathology further discussed by Innes (37).

The reason that grazing sheep are not as susceptible to phosphorus deficiency as are cattle (64, 65, 69) is no doubt due to a number of factors. Owing to the more massive skeleton of the larger ruminant, the amount of phosphorus required per unit increase in body weight is approximately double that of sheep. The growing calf thus calls for a considerably higher concentration of phosphorus in the fodder than does the lamb. Moreover, the bovine, being much less selective in its feeding habits, ingests much rank poor herbage which is usually discarded by the sheep. Carefully controlled observations on a number of experimental flocks have yielded no positive evidence that phosphate supplements exert any beneficial effects on growth or on wool production of sheep which graze on areas in Australia where the soils are singularly deficient in phosphate (67). Even on phosphorus-deficient soils, the available pasture during its actively growing period, usually provides phosphorus in excess of the requirements of sheep, which, as a rule, select the meristematic tissues to which much of the phosphate in the plant is translocated. Storage of phosphate in the skeleton during these periods provides a buffer against seasons when the phosphorus intake is low.

A considerable quantity of phosphate is secreted in the saliva of ruminants. The concentration in the mixed saliva of the sheep is usually between 20 mg. phosphorus per 100 cc. and 60 mg. phosphorus per 100 cc. and reflects in amount the concentration of inorganic phosphate of the blood (117) which, in turn, is determined by the

state of nutrition. The amount of phosphate secreted through this channel into the upper part of the alimentary tract each day under normal conditions is in excess of five times that ingested in the fodder. The efficiency of absorption of phosphorus from the ruminant intestine is, therefore, high, and any factor which might render difficult its assimilation may be expected to affect the phosphorus balance more seriously in ruminants than in other animals (64). Consideration of the capacity of calcium ions to depress the solubility of phosphate in faintly alkaline solutions might lead to the *a priori* assumption that a relatively large calcium intake in the food may adversely influence the absorption of phosphate. But while the experience of many investigators with laboratory animals leaves little doubt that a more or less balanced proportion of calcium and phosphorus in the diet is essential for normal function, and that as the  $[Ca] / [P]$  ratio deviates from the optimum value of approximately 2.0, a higher intake of vitamin D becomes essential to retain a normal physiological state, experience with ruminants does not consistently imply that untoward effects will follow the ingestion of rations in which this ratio deviates widely from what has been claimed to be the optimum. Theiler and his collaborators (104) vigorously contest the hypothesis of Marek & Welmann (57) that the ratio of calcium to magnesium to phosphorus in the diet (*Erdalkali-Alkalizität*) is of fundamental importance. From their experience with cattle in South Africa they uphold the absolute amount of these elements in the diet as the essential nutritional factor. The influence on sheep of a very considerable deviation from what has been considered the optimum *Erdalkali-Alkalizität* has been recently investigated, and the addition each day of 30 gm. of calcium and 10 gm. of magnesium, as the carbonates, to an otherwise adequate ration fed to sheep over the whole of the period from seven to twenty-one months of age, has been observed to exert no untoward effects (72). Additional vitamin D as cod-liver oil exerted no significant influence in these experiments, and it would seem that either the sun-dried straw which formed part of the ration provided an extraordinary amount of the accessory food factor, or that the ruminant requires less of it than other animals to overcome a considerable deviation from the optimum ratio of calcium to phosphorus in the diet.

The reason for the lack of agreement between the workers in Central Europe and those elsewhere may no doubt be found in the different treatment of experimental animals, for those of the Euro-

pean workers were kept in barns and supported on rations composed largely of grain and root crops, while those of the other investigators were subject to the intense irradiation of bright sunlight and fed with rations composed largely of sun-cured hay.

*Cobalt in ruminant nutrition.*—Studies of the aetiology of Coast Disease have rendered evident the importance of cobalt as a dietary constituent for ruminants.

Since the earliest settlement, graziers have observed the progressive debilitating malady which affects sheep depastured on the highly calcareous littoral of Southern Australia. Recognisable symptoms of Coast Disease have been observed to appear after experimental flocks have been confined to seriously affected areas for about four months. The demeanour of the sheep changes from the vigorous alertness of normal health to one of listlessness; their eyes become rheumy and their mucosae bloodless; their appetite fails and the lethargy and weakness progresses to a fatal termination. Autopsy reveals little other than the general findings associated with hunger oedema although haemosiderosis of the liver, spleen, and pancreas are frequently apparent. During the course of the disease the haemoglobin content of the blood has been observed to fall to less than half the normal of about 14 vol. per cent of oxygen, and in extreme cases an oxygen-carrying capacity of less than 3 vol. per cent of oxygen is often encountered (66).

Subsequent to the observation of the dramatic recovery which supervenes on the addition of minute amounts of soluble cobalt salts to the diet of sheep suffering from Coast Disease and the suggestion that similar maladies, which have been described as occurring in ruminants in other parts of the world, may have an identical aetiology (47, 63), different groups of workers have indicated that the progressive wasting syndrome evidenced by sheep and cattle when confined to a number of isolated areas in different countries is associated with abnormally low concentrations of cobalt in the soils and pastures. In Western Australia, sheep and cattle suffering from the disease described by Filmer (20) as enzootic marasmus have been demonstrated by Underwood & Filmer (110) to recover normal health after treatment with minute amounts of cobalt. Askew & Dixon (3) have described the striking improvement in the health of sheep which follows supplementing with cobalt the pastures in those areas in New Zealand where Bush Sickness is enzootic. A "Pine" affecting grazing sheep in Dartmoor (U.K.) occurs in areas

where the cobalt concentrations in the soils and pastures is considered to be abnormally low (88). The undoubted part played by cobalt in restoring and maintaining health of sheep suffering from Coast Disease, Enzootic marasmus, and Bush Sickness suggests that the "Pine" described as occurring among sheep in different parts of Great Britain (53, 27), the "Nakuruitis" affecting cattle in Kenya (86), and the "Salt Sickness" occurring among sheep and cattle in Florida (85, 4, 84), all of which evidence apparently identical symptoms, may be the result of a breakdown in the chain of metabolic events in which cobalt provides a link. Or, not unlikely, they might be of identical aetiology<sup>3</sup> and result from a metabolic disturbance caused directly from a partial or absolute shortage of cobalt in the fodder of the affected areas (67). In many of the investigations, the massive amounts of impure materials claimed to exert beneficial effects have been demonstrated subsequently to contain the minute amount of cobalt necessary.

These maladies, although strictly regional, occur in markedly different terrain, which ranges in geological formation and soil type from the aeolian sands of recent origin described by Thomas (106) to the highly ferruginous soils of Western Australia (21) and to the soils derived from recent volcanic showers described by the New Zealand workers. While many factors may be expected to influence the availability of soil cobalt to plants and hence to grazing animals, the deficiency is seemingly confined to areas in which the concentration of cobalt in the soils and in the pastures is considered to be much lower than in those of regions where ruminants suffer no untoward effects. The methods employed for the chemical estimation of the minute amounts of cobalt involved are based finally on the modification of Stare & Elvehjem (99) of Van Klooster's method (112), and therefore depend on the not altogether satisfactory expedient of matching in very high dilution the concentration of the coloured association complex of cobalt and nitroso-R-salt in the presence of a

<sup>3</sup> Since this was written, H. A. Corner & A. M. Smith [*Biochem. J.* 32, 1800 (1938)] have reported a striking benefit to supervene on dosing ewes suffering from the malady "Pine" which affects sheep in the Cheviots. These authors state that the disease may be prevented by administering cobalt, and consider that the benefit previously claimed to follow treatment with iron salts may be attributed to the presence of cobalt as an impurity in the massive doses employed. Grieg, Dryrerre, Corner & Smith [*Vet. J.* 94, 335 (1938)] have recently described the nature of the malady as it occurs in the Cheviot Hills.



reagent that is itself coloured. Even if previous chemical treatments allow quantitative recovery of the cobalt in the original materials this added colour renders many of the findings uncertain. Soils of affected regions are, however, believed to contain less than 2 p.p.m. of cobalt (39, 54). Some are supposed to contain as little as 0.1 p.p.m. (111) while healthy soils seem to range from 0.6 to 50 p.p.m. (81, 82). Affected pastures have been described to contain an average of 0.04 p.p.m. (2, 54, 111) while those from healthy regions are considered to range from 0.03 to 0.43 p.p.m. of cobalt. The grazing animal integrates the many variables of availability, selectivity, seasonal effect, etc., of which direct chemical analysis of pasture clips or of soils yields little if any information. Hence direct studies of the effect which cobalt therapy exerts in preventing or in curing untoward symptoms in animals suspected of suffering a deficiency of cobalt are far more convincing.

Association of cobalt with haematopoietic function is generally accepted. Since the observation (116) that marked polycythemia follows when a relatively large amount of cobalt is injected or fed to rats, the phenomenon has been confirmed in the rat (99, 83, 87, 11), and observed in dogs (75), mice, guinea pigs, and frogs (102), rabbits (43), and pigs (99). Although the mechanism is still obscure, studies of Orten and his colleagues have indicated that the increase in red cells and haemoglobin is the result of stimulation of the erythropoietic tissues and not due to passive accumulation as a result of a diminution of the rate of cell destruction. The evident decrease of haemoglobin and the marked morphological changes of the formed elements in the blood invariably associated with cobalt deficiency suggest that cobalt normally plays a role in haematopoietic function in ruminants (66) and the considerable haemosiderosis of the liver, pancreas, and spleen (109, 66, 81) indicate that this element may be involved in the reutilization of iron-containing residues which arise from the normal destruction of red cells. It is clear, however, that the general symptoms of lethargy and cachexia associated with cobalt deficiency in the ruminant are not primarily the result of a decreased oxygen-carrying capacity of the blood, as sheep suffer these symptoms before any anaemia is evident and, conversely, dramatically recover from them after appropriate treatment long before any marked improvement of the blood picture (66). The main effect of cobalt deficiency is seemingly on intermediary metabolism. The recent observations of Hellerman and his colleagues (31) who have described the capacity

of  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$  and  $\text{Mn}^{++}$  to reinstate activity in arginase systems suggest a possible physiological role which cobalt may assume.

Minute quantities of cobalt are involved in ruminant nutrition; the equivalent of 1 mg. of cobalt *per diem*, frequently administered, is possibly in excess of the actual requirement to continually maintain normal health in sheep depastured on regions where untreated controls all die of the deficiency within a year (66). Sheep evidently have little ability to store cobalt as correspondingly larger doses administered at intervals of a month to controlled flocks are not completely effective (46).

Ruminants, or at least sheep and cattle, are apparently unique in requiring a continual supply of dietary cobalt. Horses may be grazed for generations without untoward effects on country where sheep evidence the deficiency within four months (63) and rabbits remain well and breed when fed entirely on pasture produced on affected country (52). Coast Disease in some areas is a more complex syndrome than that of pure cobalt deficiency, and additional copper is necessary to maintain normal health (71). On these tracts, copper unaccompanied with cobalt exerts no apparent effect on the course of the malady, although the onset of symptoms of anaemia is delayed. Lambs born of ewes on such country very frequently exhibit symptoms of ataxia (66, 63, 48, 82, 70).

*Copper in ruminant nutrition.*—Sjollema (98) has described a malady, *Lechsucht*, which occurs among cattle grazing on areas recently placed under cultivation in the Eastern Netherlands. The syndrome includes depraved appetite, listlessness, and anaemia, and is corrected by the addition of copper to the diet. The copper content of hay produced on the affected areas is from 1 to 3 mg. per kg. while that of normal hay varies from 6 to 12 mg. per kg. Symptoms in oats and other plants (*Ontginningsziekte*, *Urbarmachungskrankheit*, *Heidemoorkrankheit*, Reclamation disease), which are now generally regarded as being characteristic of copper deficiency, are prevalent in the areas where *Lechsucht* occurs. The pathological condition in both the plants and the grazing cattle has been controlled by the application of 50 to 100 kg. of copper sulphate per hectare.

A disease characterised by incoordination of gait which appears in young lambs soon after birth has been observed in Great Britain (101) where it has been described by many local names, mainly as Swayback or Warfa; it also occurs in Peru (25) where it is referred to as *Rengeurra*; it occurs in Sweden (55) and in South Africa (16);

it is also known in Australia where it occurs in many localities (9, 66) and where it is at times associated with Coast Disease (66, 82, 13). Similarity of the described symptoms and the pathological findings leaves little doubt that it is a clinical entity. The symptoms are essentially those of spastic diplegia which seems invariably associated with degenerative changes in the spinal cord (101, 38, 13, 9) and at times with diffuse symmetrical demyelination of the cerebral white matter (38, 9) which often results in the formation of porencephalic cavities as a sequel to gross destruction in the brain.

Bennetts & Chapman have demonstrated that the malady as it occurs in Western Australia may be prevented by supplementing the diet of gestating ewes on affected country with 15 mg. of copper per day. The copper content of the blood and organs of ataxic lambs has been observed to be very materially reduced, the liver copper being diminished to about a twentieth of normal (10) which is of similar magnitude to that observed in sheep grazing on the littoral where both cobalt and copper deficiency is experienced by ruminants (81). Blood copper is reduced to approximately one-tenth of the normal value in ewes on both types of country (13, 81). Associated with the anaemia, polychromasia, and punctate basophilia of the red cells are a constant finding (38, 10, 66).

Experimental observations in South Australia (71, 68) leave little doubt that the ataxia of young lambs which is associated with Coast Disease has its origin in the copper deficiency which accompanies the shortage of cobalt in some areas. Appropriate treatment of sheep on these tracts with cobalt or with copper reveals the uncomplicated syndrome of either deficiency. Where the complex malady occurs lambs born of untreated ewes usually expire of cachexia without evidencing ataxic symptoms. Copper deficiency in plants growing on those areas has been observed (92).

Dunlop & Wells have recently described a large scale experiment conducted on a number of areas in North Derbyshire, England, where the intensity of Swayback had been previously observed to vary. By supplying salt licks containing 0.3 per cent of copper to groups of gestating ewes distributed over the whole of the affected region, the incidence of ataxia in their lambs was reduced from 15 per cent to 1.8 per cent (15).

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# IMMUNOCHEMISTRY

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Several books and reviews relating to immunochemistry appeared during 1937 and 1938 (1 to 5). Special attention may be drawn to a second edition of Marrack's book (1), *The Chemistry of Antigens and Antibodies*, which deals extensively with general problems of immunochemical reactions [see also (2)], and to the proceedings of the immunochemical section of the Second International Congress for Microbiology (6).

## PROTEINS

*Toxins.*—Recent extensive work on the purification of toxins has led to the separation of several of these in a practically pure state, and they appear indeed to belong in the class of proteins. A comprehensive review on bacterial toxins has been presented by Eaton (7).

The purification of diphtheria toxin has been simplified by the use of special protein-free media, permitting its separation by ammonium sulfate fractionation and adsorption of impurities (8), or ultrafiltration (9). By the method of isoelectric fractionation, Eaton (10) obtained a preparation containing only 0.5 to 2 per cent of contaminating bacillary protein and having an optical rotation of about  $-45^\circ$ , which was not appreciably changed by modification to toxoid. Purified diphtheria toxin gives a strong Sakaguchi test and the amino nitrogen value, "unusually high for a protein," is 14 per cent of the total nitrogen. Pappenheimer's preparation (8), closely similar in activity to Eaton's and behaving as a typical protein, contained 0.00046 mg. nitrogen per Lf unit, and had 80,000 M.L.D. per mg. nitrogen. The following percentages of various constituents have been found (11): N, 16.0; S, 0.75; tyrosine, 9.0; tryptophane, 1.4; histidine, 2.3; arginine, 3.8; lysine, 5.3; the molecular weight is estimated as 14,000 to 18,000; isoelectric point, pH 4.1;  $[\alpha]_D = ca. -40^\circ$ . In agreement with previous results of Goldie with diphtheria (and tetanus) toxin (12; cf. also 179), short acetylation with ketene which involves the  $\epsilon$ -amino groups of lysine destroys its toxicity but not the capacity to flocculate with antitoxin; however, the latter is



abolished upon further treatment. The conditions for converting purified diphtheria toxin into toxoid have been determined for formaldehyde, acetaldehyde and derivatives thereof by Eaton [13; *cf.* 14 and (for the chemistry of anatoxin formation) 15]. An anatoxin (and toxin) of activity equal to the preparations mentioned above has been separated by Boivin (16) by precipitation with trichloroacetic acid. Anatoxin obtained by Theorell & Norlin (17) with repeated acetone precipitation and cataphoresis appeared to be a homogeneous protein when tested electrophoretically in the apparatus of Theorell. Several papers treat of inactivation of bacterial toxins by various agents: salvarsan (18), lipids and sterols (19), carbon disulfide and mustard oil (20), and ascorbic acid (21). The inactivation of *Pneumococcus* hemolysin by sterols has been studied by Cohen *et al.* (22); it is concluded that inhibition depends upon hydroxyl groups and a double bond in the sterol; the thiol grouping in the lysins is not blocked by cholesterol (*cf.* 19).

*Viruses.*—In several cases the crystalline virus proteins are now regarded as nucleoproteins (*cf.* 23), as is the bacteriophage preparation of Northrop (24). The heat-stable S substance (Craigie) from vaccinia has been purified by Parker & Rivers (25) by fractionation with ammonium sulfate, precipitation by alcohol and by acid, and boiling at various reactions to remove heat-coagulable proteins. The material appears to be a protein (N, 16.5 per cent), soluble in water and acidified 80 per cent alcohol, and giving a positive Molisch test. It is antigenic, and reacts also with an immune serum prepared with boiled elementary bodies. Reviews on the serology of viruses have been written by various authors (26 to 29).

*Bacterial proteins.*—A study of the constituents of tuberculin was carried out by Seibert, Pedersen & Tiselius (30) by means of the ultracentrifuge and electrophoresis. From culture filtrates a practically uniform protein with a molecular weight of 32,000 was isolated which proved to be highly active in eliciting local anaphylactic reactions and in causing death in tuberculous guinea pigs. Also, substances of lower molecular weights (16,000 and 9,000) having a low nucleic acid content and active in producing skin reactions were isolated from old tuberculin. Maschmann (31, *cf.* 31*a*) has reported the separation of tuberculin into two fractions, one responsible for the skin reactivity, the other for systemic reactions. The former is a substance of protein nature, and is broken down by papain or trypsin, not by cathepsin, while the other is resistant to proteolytic enzymes.

Menzel & Heidelberger (32) isolated proteins from human, bovine, and avian tubercle bacilli and the timothy-grass bacillus. In each case several fractions were obtained differing chemically and serologically, *e.g.*, the human strain contained at least three antigenic proteins. Corresponding proteins from the various microorganisms showed differences.

Studies on the antigens of hemolytic streptococci have been reported by Stamp & Hendry (33) and Mudd and his associates (34). The latter, working with streptococci of group A (Lancefield), have encountered new antigenic substances, designated labile antigens, and engendering both type- and species-specific antibodies. They were extracted from streptococci disintegrated by ultrasonic vibration or grinding of the microorganisms in a frozen or dried state, the material finally being purified by acid precipitation and solution in fifty to seventy per cent alcohol. The antigen has now been found by Sevag *et al.* (35) to be a nucleoprotein. The instability of the substance—its activity deteriorates quickly and is destroyed on heating at 56° C—depends in part on oxidation, but if this has not gone too far restitution is possible by reducing agents (cysteine or thioglycolic acid). Upon acid hydrolysis the substance yields two serologically distinct components, one group-specific, the other type-specific. Treated with sodium bisulfite, it acquires hemolytic properties which can be neutralized by immune sera; this lysin is very similar to the known oxygen-labile streptolysin (*cf.* 36). Ultracentrifugation shows that the antigen has a molecular weight of about 100,000 and is essentially homogeneous.

With a crude nucleoprotein fraction, not coagulable by heat and containing 4 per cent phosphorus, liberated from S or R pneumococcal cells during controlled autolysis in which the cells became Gram-negative, Dubos *et al.* (37) were able to develop protective antibodies in rabbits against virulent Pneumococci of several types; there is a resemblance to the "pneumococcal species antigen" of Day & Harley.

In Fusobacteria, Weiss & Mercado (38) ascribe the type-specificity to protein, as had been ascertained by Lancefield for certain streptococci.

*Animal proteins.*—Newer studies have emphasized the complexity of blood serum as to its protein constituents. Thus with a significantly improved electrophoresis apparatus, Tiselius has succeeded in distinguishing three globulin fractions in normal serum [*vide* "Antibodies," p. 593; *cf.* (39)]. Hewitt (40) has described a new globulin,

rich in carbohydrate and having a low tryptophane content (0.8 per cent), called "globoglycoid." A fraction of normal human serum, termed  $\alpha$ -globulin, was isolated by Kendall (41; *cf.* 46); it was shown with the aid of immune serum to behave like a pure protein. The remaining globulin contained more than one antigen. The author used precipitation with absorbed immune sera to determine quantitatively the amount of the globulins in the serum of pathological cases. A study by Marrack & Duff (42) of the water-soluble and -insoluble globulins (*cf.* 43) by means of an immune serum to whole serum globulin would indicate that "these fractions are not present, as such, in the whole globulin." The polysaccharide isolated from horse pseudoglobulin was found by Coghill & Creighton (44) not to be precipitated by anti-horse globulin serum and not to inhibit the reaction of such sera, the experiments failing to support the assumption that the polysaccharide plays a rôle in the specific reactions of the serum protein.

From the usual preparations of crystallized serum albumin, Hewitt (40, 45) has separated a hitherto unrecognized serum protein, "seroglycoid," which has a high polysaccharide content, is not heat coagulable and is not precipitated by two per cent trichloroacetic acid, and a crystalline serum albumin, "crystalbumin," which is practically free from carbohydrate and constitutes about 85 per cent of the entire albumin fraction; serological reactions of these proteins are described (46). Kekwick (47) has likewise obtained two fractions from crystallized horse-serum albumin, both secured in crystalline form. Besides these, serum albumin possibly contains non-dialysable material of high carbohydrate content (*cf.* 47*a*). His carbohydrate-poor albumin (*ca.* 0.08 per cent carbohydrate) is probably identical with Hewitt's crystalbumin; the other albumin had 1.95 per cent carbohydrate. Both proteins were antigenic, and although very similar in physicochemical properties, could be distinguished serologically despite overlapping reactions.

Evidence by Kabat & Heidelberger (48) is offered to the effect that in horse-serum albumin, unlike egg albumin, tyrosine (and perhaps histidine) is not involved in the reaction with antibody, since immune sera against serum albumin react quantitatively alike with horse-serum albumin and an azoprotein derivative thereof, whereas antioalbumin serum does not react with the corresponding azoprotein made with egg albumin. "The structural pattern of the two proteins therefore seems to differ very widely."

Keratin from human hair, wool, and feathers, brought into solution by the action of reducing agents (Michaelis & Goddard), was investigated by Pillemer & Ecker (49; *cf.* p. 592) with the precipitin reaction. Antisera to the reduced keratins gave specific reactions under certain conditions. That the class of protamines also is now open to serological study would appear from a recent paper: Gutman (50) reports that she was able to confer antigenic activity on clupein by combining this protamine with phenylisocyanate. The immune sera obtained gave precipitin reactions with the antigen used, divers phenylureido proteins and clupein itself. (A special technique for the reaction was employed because of the precipitation of proteins by clupein.) In the author's opinion the antigenicity of phenylureido-clupein is due to the introduction of aromatic rings. In a study of some nucleoproteins, no antigenic effects were observed (50a).

By means of the method of specific inhibition Mutsaers (51) has clearly demonstrated that the serological specificity of xanthoproteins is due to the nitrotyrosine grouping. Using numerous related compounds, the author found that the reactivity depends upon the presence of nitro and hydroxyl substituents in the benzene ring and a carboxyl group, free or esterified. No cross-reactions were observed between nitroproteins, and azoproteins made from picramic acid and some other amino nitro compounds. Nitration of gelatin did not confer antigenic properties upon this protein, and nitrogelatin was not precipitated by immune sera to other nitroproteins. The serological similarity of nitro- and diazoproteins has been confirmed in the case of gelatin by inhibition tests (52). Reports have been made of serological investigations on proteins treated with ninhydrin (53) and changes in protein specificity by use of high pressure (54).

A resumption of the serological investigation of the assumed antigenicity of plasteins became desirable in view of Folley's study indicating a molecular weight not higher than 1,000, which would render antigenic capacity improbable. Experiments by Flosdorf & Mudd (55) led them to the opinion that the antibodies found on injection of plastein are due to traces of denatured proteins and of enzymes contained in the injected material.

*Enzymes, complement.*—By the isolation of enzymes in the form of their crystallized proteins, their serological investigation has been put upon a firmer basis.<sup>1</sup> The specificity of pepsin of various animals

<sup>1</sup> See also TenBroeck; Kirk & Sumner [*Ann. Rev. Biochem.*, 4, 570, 580 (1935)] and a review by Sumner (56).

was investigated by Seastone & Herriott (57) by means of precipitin sera from rabbits injected with crystallized (Northrop) swine pepsin or pepsinogen. The anti-pepsin serum precipitated swine and bovine pepsin equally well, much less that of guinea pigs and not at all pepsin of rabbit, chicken or shark. The observation that the species-specificity of anti-pepsin sera is less sharp than that of antibodies for serum proteins is attributed to the use of alkali-denatured pepsin, in analogy to the behavior of denatured proteins in general. Pepsin antisera cross-react with pepsinogen whereas antisera for pepsinogen do not act upon recrystallized pepsin. Oxidized urease, according to Pillemer *et al.* (58), incites antiurease production, as does urease, in contrast to preparations irreversibly inactivated by irradiation. It is suggested that in the former, sulfhydryl groupings, significant for antigenic capacity, are oxidized to disulfide groupings.

In studies on complement it was noticed by Ecker and colleagues that a correlation exists between the concentration of ascorbic acid and the complement activity of guinea-pig sera. The interesting observations are further reported (59) that complement inactivated under controlled conditions by oxidants or ageing can be reactivated by means of reducing agents, hydrogen sulfide, sodium hydrosulfite or ascorbic acid, and similar observations were made on complement reduced in activity by phospholipids or by iodoacetic acid (60). The effects are attributed to oxidation and reduction of sulfhydryl groups (*cf.* 61). Sedimentation of complement and  $\beta$ -lysin in the ultracentrifuge has been studied by Gratia & Goreczky (62). Partial extraction of lipids from dried complement did not reduce the activity (63).

*Hormones.*—The formation of antibodies to hormones (Collip) in the immunological sense is not difficult to comprehend in the case of hormones of protein nature and of molecular weights akin to those of common proteins. In fact antibodies have been produced to thyroglobulin (196), and clinical observations and anaphylaxis experiments in animals seem to indicate the occurrence of antigenic effects with insulin (64). The reason why insulin appears to be a rather poor antigen (*p.* 591) has not been established.

As regards gonadotropic hormones, Brandt & Goldhammer (65) concluded that their complement-fixing sera, produced by means of these hormones, are directed against contaminating substances, not against the hormone itself (66; *cf.* 67). However, antihormonal activity was demonstrable in the sera after treatment with hormones from a foreign species, and species-specificity was noted (*cf.* 68).

Zondek & Sulman (69) also demonstrated species-specificity of their "antigonadotropic factor" produced in rabbit and rat serum; they likewise found differences between human prolans from pregnancy urine, pregnancy blood, and pituitary. Prolan, completely heat-inactivated, did not give rise to antihormones (70). Rowland's results (71), while not uniform when various antigonadotropic sera were used, still indicate the existence of species and source ("organ") specificity in the antihormone reactions. Experiments on antithyrotropic sera are in general accord (72, 72a); Eichbaum *et al.* (73) described complement-fixing antibodies to the thyrotropic hormone. Further papers to be cited deal with the antihormonal (or augmenting) power of sera against pituitary extracts (74), the mechanism of thyrotropic action (75), and with sera which counteract the effect of thyroglobulin (148).

#### HAPTENS. CARBOHYDRATES, LIPIDS

An outstanding result among the investigations on bacterial haptens is that of Ivanovics & Bruckner (76, 146) who isolated a unique polypeptide as the specific capsular substance of *B. anthracis* and *B. mesentericus*, demonstrating the existence of a new sort of bacterial hapten. It was found to be an acid ( $[\alpha]^{20}_D = ca. +21^\circ$ ) and was purified by preparing its copper salt, then the silver or mercury salt. Upon acid hydrolysis it yielded the optical isomer of the common *l*(+)-glutamic acid (*cf.* p. 591). The substance is considered to be a high molecular polypeptide containing only the one amino acid, of which there are, presumably, forty to fifty residues in the molecule. This estimate is tentative because full purity could not be ascertained, and the authors point out that the acid equivalent is higher than would follow from a regular polypeptide structure. It is suggested that the capsular material makes for virulence of the organism since it is built up from the "unnatural" *d*(—)-acid and therefore would be resistant to digestive enzymes.

*Bacterial polysaccharides.*—The methods devised to isolate bacterial polysaccharides in an unaltered state have been applied by Heidelberger and coworkers (194) to the polysaccharide of *Pneumococcus* VIII, analytical data being given, and to *Pneumococcus*-I carbohydrate by Chow (77), the latter preparation apparently being weakly antigenic in rabbits [*cf.* Downie, immunity in mice (78)]. Preparations of the polysaccharides of *Pneumococci* I, II, and III were made by Wadsworth & Brown (79) by ultrafiltration of cultures

grown in infusion-free peptone medium; the percentage of nitrogen in the Type-II preparation was 1.4 to 2.0, and in Type III 0.9 to 1.5. A polysaccharide isolated from *Pneumococcus* I by Sevag's method (80) appeared to be a complex possessing the functions of both the type-specific polysaccharide and the species-specific carbohydrate "C"; a non-antigenic substance, it contained 6.35 per cent N, 2.78 per cent P, and 23.0 per cent reducing sugar. From *Pneumococcus*-I polysaccharide Felton & Prescott (81) obtained a material with increased antigenicity (for mice) by treatment with hot dilute sodium hydroxide, and subsequently with ammonia, from which the authors conclude that the acetyl groups are not requisite for immunizing activity. Carbohydrate-containing preparations of much higher immunizing capacity in mice than demonstrable in the pneumococcal cells from which they were derived were described by Felton (82). The aldobionic acid in the polysaccharides of *Pneumococci* III and VIII was shown by Hotchkiss & Goebel (83) to be glucose-4- $\beta$ -glucuronide, and the synthesis of two aldobionic acids and of several aldobionides has been reported (84).

The specific carbohydrate of *Pneumococcus* XIV, having especial interest on account of the findings of Finland & Curnen (85) that horse immune sera for this microorganism contain agglutinins for human blood, has been studied by Hoagland, Beeson & Goebel (86). The substance resembles in some respects the group-A substance from pig stomach and in fact the latter is precipitated, in the cold, by Type-XIV horse antiserum. [For a comprehensive review on *Pneumococci*, see (87).]

Fuller (88) has described a method for rapidly preparing group-specific polysaccharide extracts from hemolytic streptococci (and type-specific extracts from pneumococci) by dissolving the bacteria in formamide at 150° C, which serves to release the polysaccharide from combination with other substances. Kendall, Heidelberger & Dawson (89) isolated a polysaccharide from group-A hemolytic streptococci, consisting of N-acetylglucosamine and glucuronic acid units; it was found by Loewenthal (90) to behave as a hapten. For carbohydrates in subgroups of group-B hemolytic streptococci, see Lancefield (91).

Heidelberger & Menzel (92; *cf.* 112) have described the fractionation of the complex mixture of polysaccharides obtained from the tubercle bacillus, yielding besides inactive polysaccharides (*cf.* 93) two which are immunologically active (*cf.* 94, 113); one has  $[\alpha] =$

+100.9°, is low in pentose, and appears to contain magnesium palmitate in chemical combination; the other,  $[\alpha] = +27.1^\circ$ , has a relatively high pentose content but is difficult to separate from the former. The carbohydrates "are built up chiefly of *d*-arabinose and *d*-mannose units in varying proportions." Tubercle bacillus polysaccharides were separated cataphoretically by Seibert *et al.* (30).

Morgan (95) has given closer study to the polysaccharide of *Shigella dysenteriae*. It is found to contain 98 per cent reducing sugar, with 15 per cent *d*-galactose, 7.5 per cent *l*-rhamnose, about 20 per cent hexosamine, and 5 per cent acetyl apparently bound to nitrogen.

A preparation of a *V. cholerae* (Group VI) polysaccharide was obtained by Shrivastava & Seal (96) with avoidance of drastic chemical treatment (N, 2.62 per cent); glucose was identified after hydrolysis. In a study of a vibrio strain, Linton *et al.* (97) reported that the polysaccharides differed in chemical and serological properties according to the nature of the media, and likewise, differences were seen among variants of *V. cholerae* derived from a single-cell strain.

In *Salmonella* bacilli containing Factors IV and V, the latter are very probably contained in a single molecule [Meyer (133)].

Upon treatment of the insoluble carbohydrate fraction of yeast with hydrochloric acid, several polysaccharide fractions were secured (98), of which one, containing an acid group, appeared to be the substance serologically related to the specific polysaccharide of *Pneumococcus* II.

Other papers relating to polysaccharides of microorganisms may be quoted: gonococci (99); "*Bacterium typhi flavum*" (100); *Klebsiella rhinoscleromatis* (101); *Cl. welchii* (102); *Asterococcus mycoides* (103); *C. diphtheriae* (103a); molds (a review) (104).

A possible source of error in the work on bacterial polysaccharides has been pointed out by Goebel (105), namely contamination with the A substance, rich in carbohydrate, which is present in pig stomach and consequently in commercial peptones used for culture media. Chemical data on the substance are presented.

A "stable" carbohydrate-containing hemolysin-leucocidin was isolated from  $\beta$ -hemolytic streptococci by Czarnetzky, Morgan & Mudd (106; *cf.* p. 581). It does not form antibodies but is precipitated (not neutralized) by antisera produced with whole streptococci. The analytical data and molecular weight determination of the substance



correspond to  $C_{75}H_{148}O_{71}N_3P$ . On heating with alkali a crystalline substance (molecular weight 720) was obtained as a sodium salt which retained a considerable part of the original hemolytic and leucocidic activity but no longer reacted with immune sera. This substance was free from nitrogen and phosphorus. It was toxic for mice and rabbits.

*Other carbohydrates.*—A paper by Marrack & Carpenter (107) presents an interesting study of the precipitin reactions of Type-II antipneumococcus sera with various vegetable gums after mild hydrolysis. The reactions of the gums, with one exception (namely flax mucilage which does not contain glucuronic acid), and also to some extent the reactions of the pneumococcus carbohydrate, are specifically inhibited by glucuronic acid, glucuronides, and some other derivatives of glucuronic acid, which indicates that this acid is a main determinant group. Other reports are concerned with sera for gum arabic (108) and pectin (108a).

Uhlenhuth & Remy, who previously failed to obtain antibodies to glycogen, now report (109) having got immune sera by injecting glycogen adsorbed to alumina; despite the fact that the glycogen probably contained protein the authors consider that the sera reacted with the glycogen itself. Campbell (110) obtained antisera against glycogen from fresh-water clams but not against glycogen from the livers of vertebrates; the same author was able to use the carbohydrates of various helminths for their serological differentiation (111).

*Lipids.*—Macheboeuf and his colleagues (112) found the haptenic lipid fraction from tubercle bacilli to be a mixture (chiefly in the form of magnesium salts) consisting of high molecular fatty acids combined by ester linkages with either glycerophosphoric acid or inositol-monophosphoric acid. The preparation lost its serological activity progressively as the fatty acids were liberated with dilute mineral acid. Which of the two sorts of complex acids carried the haptenic property is unknown. A small amount of polysaccharide is considered as a contamination. The chemical analysis of the lipids of tubercle (and leprosy) bacilli has been continued in Anderson's laboratory (113); among the fatty acids, which in part have branched chains, an acid ( $C_{29}H_{58}O_2$ ) was separated and characterized by its tribromoanilide [Wagner-Jauregg (114)] and a saturated hexoxy, methoxy acid (mycolic acid) of 1,284 equivalent weight was isolated by Anderson *et al.* (115).

With reference to the monograph of Brunius (116) on the Forssman substance in horse kidney, it is to be added that besides hexosamine there is another carbohydrate, probably a hexose. The nitrogen content varied between 2.0 and 2.3 per cent; the best purified substances had 59.5 to 63.8 per cent carbon; the small quantities of sulfur and phosphorus present were probably due to impurities. The activity was lost upon treatment with diazomethane but was unaffected by nitrous acid, phenylisocyanate, and by proteolytic enzymes. After inactivation by alkali, the activity could not be restored by ketene.

Additional papers are concerned with: anticholesterol sera (117) and antisera to commercial lecithin (118), reactions of normal sera with a substance in commercial lecithin (119), Wassermann substance (120), alleged antigenicity of vitamin D (121), possible anaphylactogenic activity of oleyl-N-methyltaurine (*cf.* p. 601).

#### ANTIGENIC CARBOHYDRATE-LIPID COMPLEXES

The discovery by Boivin & Mesrobian, and by Raistrick & Topley, (in 1933) of carbohydrate-lipid complexes in the "smooth" forms of Gram-negative bacteria, a class of highly antigenic labile substances which incite the production of agglutinins of the "granular" or O type and which are endotoxins, has prompted the examination of many bacteria.<sup>2</sup> Recent studies resulting in the isolation of such complexes have been pursued with dysentery bacilli (124, 125, 126), various species of *Pasteurella* (127), "*B. pyocyaneus*" (128), *B. anthracis* (129), *Eberthella typhosa* (130 to 134), etc. In the case of *Proteus* X<sub>19</sub>, the carbohydrate-lipid antigen appears to represent the material responsible for the Weil-Felix reaction (135). From *V. cholerae*, antigenic carbohydrate-containing substances, belonging probably to this class, and showing serological complexity, have been prepared by White (136; *cf.* 137).

For the isolation of the substances, in addition to the trichloroacetic acid technic of Boivin, tryptic digestion (130, 131) and extraction of the dry cells with diethyleneglycol (125, 134) have been utilized. These high-molecular compounds (nitrogen, 2 to 3 per cent) have been characterized (122) as consisting of a combination of polysaccharides with fatty acids and also with acetic and phosphoric acids,

<sup>2</sup> For more detailed information, the reader is referred to reviews by Boivin (122, 123).

the polysaccharides representing one-half to two-thirds, the fatty acids one-fifth to one-quarter of the complex. The polysaccharide portion contains simple sugars, amino sugars, and frequently uronic acids.

Morgan (125) has made a careful analysis of the antigenic complex from *Shigella dysenteriae* as secured with his diethyleneglycol method. This substance, which engenders both antibacterial and heterophile antibodies, and has the properties of Shiga endotoxin (*cf.* 124), yielded the following fractions upon being heated in 0.1 N acetic acid: the specific polysaccharide (95) previously isolated by him (48 per cent), lipoidal substances (10 per cent), an acid-insoluble material (17 per cent) with 10.2 per cent nitrogen and 1.1 per cent phosphorus, and diffusible substances including inorganic phosphate (14 per cent). The heterophile activity of the complex is, according to Meyer (126), considerably weaker than that of the liberated polysaccharide as was also found for a substance from *S. schottmülleri*.

Studies on virulent typhoid organisms (130, 132; *cf.* 133) have resulted in distinguishing two carbohydrate-lipid complexes, corresponding to the somatic antigens O and Vi, which differ in carbohydrate content and in precipitability with certain reagents such as uranium acetate (*cf.* 138). A similar coexistence of two immunologically distinct antigens has been observed in some other cases (127, 138), *S. paratyphi C* and certain *Pasteurellas*.

Concerning the debated nature of the specific precipitable substance in *Brucella* bacteria, recent investigations have led to the separation of antigenic materials which, besides fatty acids and relatively small amounts of carbohydrates, contain amino acids. In the "end-antigen" of Pennell & Huddleson (139), about a third of the nitrogen value of 5 to 10 per cent is represented by tryptophane and tyrosine. Results broadly similar were found by Pop *et al.* (140) with a complex obtained by Boivin's method. For lipid-polysaccharide compounds in tubercle bacilli, *cf. Ann. Rev. Biochem.*, 7, 495 (1938) and this volume p. 145.

In connection with the attempts, thus far futile, to isolate the pneumococcal antigen responsible for the development of type-specific antibodies in the rabbit (*cf.* 141), it may be mentioned that, in addition to an autolytic enzyme in the bacteria themselves (37), heat-stable nucleases exhibiting maximum activity at 70° C which inactivate the "capsular polysaccharide antigen" without decomposing the specific polysaccharide (and render the cells Gram-negative) have

been isolated by Dubos & MacLeod (142) from polymorphonuclear leucocytes, certain animal tissues, and pancreatin (*cf.* 143).

#### SYNTHETIC CONJUGATED ANTIGENS

Goebel & Hotchkiss (144) have synthesized azoprotein antigens containing respectively glucuronic acid and galacturonic acid; these are serologically sharply differentiable from one another and from antigens containing glucose or galactose. The galacturonic acid azoprotein is precipitated by antipneumococcus sera, which in the case of Type I is in accord with the established presence of galacturonic acid in the polysaccharide. [For the reactions of glucuronic acid antigens with antipneumococcus sera, see *Ann. Rev. Biochem.*, 6, 630 (1937); *cf.* p. 588.] Remarkable is the fact that antipneumococcus horse sera react with quite unrelated azoproteins containing benzene-sulfonic or carboxylic acids. In continuation of this work, conjugated antigens containing glycosides of cellobiose and cellobiuronic acid have been prepared (145). An antiserum to the latter gives precipitin reactions not only with antigens made from glucuronic acid but also with those containing glucose and cellobiose; in addition, it precipitates an azoprotein made from the capsular polysaccharide of Type-III Pneumococcus. The cellobiuronic acid antigen is precipitated by antipneumococcus sera for Types III, VIII, and—unexpectedly—II, but not by those for Type I, and it is noteworthy that the sera for this antigen protect mice against infection with Pneumococci II, III, and VIII. Another instance where synthetic conjugated antigens interact with antibacterial sera has been provided by Ivanovics & Bruckner (146), in this case with a non-polysaccharide hapten. The authors found that sera reacting with the capsular hapten of anthrax bacilli (*cf.* p. 585) specifically precipitate azoproteins prepared from *d*(—)-glutamic acid. Immune sera for the latter did not precipitate the capsular substance, the suggestion being made that of about fifty glutamic acid residues in the molecule only the terminal one would combine with the antibody.

A new method has been developed by Clutton, Harington & Mead (147) for the introduction of carbohydrate groups into proteins, namely by means of azides such as O- $\beta$ -glucosido-N-carbobenzyloxy-tyrosine. Removal of the carbobenzyloxy group was achieved with the aid of sodium in liquid ammonia or by reduction with a large amount of palladium. Antisera against gelatin and insulin conjugates did not

precipitate the homologous antigens but gave definite reactions with a globulin conjugate, and both the gelatin and insulin antigens were precipitated by the antiserum to the globulin conjugate. These observations may be compared with those of Wormall, and Hooker & Boyd. The precipitin reactions were inhibited by substances possessing a  $\beta$ -phenolic glycoside linkage (O- $\beta$ -glucosido-tyrosine, arbutin, or salicin).

Likewise by means of azides, N-carbobenzyloxy-3,5-diiodothyronyl residues have been introduced into proteins (148); iodination of the product converts the diiodothyronyl residues into thyroxyl groups, at the same time replacing the tyrosine of the original protein by diiodotyrosine. The specificity of the resulting thyroxylproteins is determined in part by the thyroxyl and in part by diiodotyrosyl groups; the antisera give precipitin reactions with thyroglobulin. It is interesting that injection of the antisera into normal animals causes a resistance to the action of administered thyroglobulin and thyroxine (*cf.* 72a).

In elaboration of the findings of Berger & Erlenmeyer, precipitin and inhibition tests have been made (149) with antisera to pyridine compounds, viz., 3-amino pyridine, 3-amino-4-carboxy pyridine, and 4-amino-3-carboxy pyridine. The results as regards specificity were in satisfactory agreement with those obtained with benzene derivatives. The serological properties of pyrazolone derivatives, investigated for the first time by Erlenmeyer & Berger (150), have been studied by Harte (151).

Conjugated proteins were also prepared [Pillemer & Ecker (152)] by the interaction of sulfhydryl groups of keratine with various halogenated fatty acids, bromoethylbenzene and benzyl chloride, and their specificity examined.

The fact that certain azo dyes give precipitin reactions with immune serum to the corresponding azoproteins and produce anaphylaxis in sensitized guinea pigs (Landsteiner & van der Scheer) has been reinvestigated (153) on account of criticisms by Fierz-David *et al.* (154; *cf.* also p. 600) and the previous results have been confirmed, also by means of the reactions with the isolated uterine horns from sensitized guinea pigs. The latter method proved useful for demonstrating anaphylactic effects with azo dyes which gave no or only faint precipitin tests, *e.g.*, tartranilic acid-resorcinol dye.

Phenylureido derivatives of protamines have been investigated by Gutman [50 and p. 583] and Creech & Franks (155) studied the

precipitin reactions of conjugate proteins containing the carcinogenic hydrocarbon 1,2,5,6-dibenzanthracene. With animals sensitized by an azoprotein made from *p*-aminophenylarsenic acid, Fierz, Jadassohn & Stoll (156) observed that the anaphylactic reactions are inhibited by a corresponding dye containing the  $-N=N-$  linkage (as present in the azoprotein) but not by dyes having an  $-NH-N=$  grouping (quinone-hydrazone form).

#### ANTIBODIES

*Properties and purification.*—Studies have been continued, with physicochemical methods, of the globulin components carrying the antibody function.<sup>3</sup> With his recently devised electrophoresis method Tiselius (159) has found that rabbit antibody (to egg albumin) is associated solely with the fraction designated as  $\gamma$ -globulin (isoelectric point pH 6.0), which in lesser amount is present in normal rabbit serum. A concentration to 85 per cent antibody globulin could be effected by the electrophoretic method. In horse serum, Pneumococcus-I antibody appears to be a special globulin migrating between  $\beta$ - and  $\gamma$ -globulins; the corresponding boundary in the electrophoresis experiment disappears from the serum upon removal of the antibodies by absorption with the polysaccharide (160). The molecular weights of Types I and III pneumococcal antibodies, purified by dissociation of specific precipitates or "agglutinates," have now been found in Svedberg's laboratory to be about 930,000 for horse, cow and pig antibody and 157,000 for the rabbit and monkey (161). From the high molar frictional constants determined on these globulins, it is supposed that "the heavy antibody molecule is neither compact nor spherical." In ultrafiltration experiments, Went & Sarkady (162) could not differentiate the antibodies from other globulins in rabbit immune sera as proteins of large particle size. By means of immune sera produced to toxoid-antitoxin floccules or specific precipitates of pneumococcal carbohydrates, antitoxic and antibacterial immune bodies were distinguishable [(163); cf. 164]; experiments on differences in precipitin reactions of normal and antibody globulin were

<sup>3</sup> With regard to the comparison between horse and rabbit antisera, see *Ann. Rev. Biochem.*, 6, 632, 633 (1937); also reference (157). On the ultracentrifugation of antibodies, cf. also (158) and (62). To some extent at variance with experiments on ultracentrifugation are ultrafiltration results on the aggregation and dispersion of antibodies, as presented in papers appearing after the completion of this manuscript (158a).

presented by Marrack & Duff (42). Also Lourau-Dessus (165) reports differences between normal serum proteins and antibodies in electrophoresis experiments.

Studying antibodies as surface films, Danielli, Danielli & Marrack (165a) found that these films are not able to combine with homologous polysaccharide, which suggests that the combining groups are "not prosthetic groups nor combinations of amino acids which retain the arrangement conferring the specific properties when the protein is unrolled."

Heidelberger *et al.* (166) have extended to additional cases the method of separating pneumococcus antibodies by dissociation of immune precipitates in 15 per cent salt solution or upon treatment with barium hydroxide (*cf.* 167). With Type-I antipneumococcus sera, the dissociation of specific agglutinates (*cf.* 168) appeared to be the expedient method, leading to the recovery from Pneumococcus-I rabbit serum of two-fifths of the total antibody content in the form of solutions containing 88 to 97 per cent of reactive antibody. (A polyvalent bovine antiserum was fractionated into Types I, II, and III antibody of high purity.) Practical results in the concentration of antitoxin have followed from the use of digestive enzymes (see below). On the effect of increased salt concentrations on the amount of precipitates, *cf.* 168a.

Doladilhe and colleagues (169) report the separation of divers antibodies (and complement) in a special globulin fraction, designated as *protéine visqueuse*; by further purification the antibody was obtained as a water-soluble protein which, according to the authors, can be differentiated from normal serum proteins by anaphylactic experiments (*cf.* 170).

Regarding the properties of antibodies, the question of their enzymic digestion has been taken up again. Rosenheim (171) found that, while O antibodies in antityphoid horse serum were always destroyed, H antibodies, surprisingly, acquired a resistance to pepsin and trypsin in the later stages of immunization; the possible bearing of this result on the structure of antibodies is discussed. From the studies of Pappenheimer & Robinson (202), Pope (172), and Weil *et al.* (173) [*cf.* 174, 175], it appears that diphtheria antitoxin can be digested to some extent without destruction of the antibody function. In experiments of Chow *et al.* (176) with purified Pneumococcus-I precipitin, there was a parallelism between protein degradation and the destruction of antibody (*cf.* 176a).

The effect of formaldehyde on antisera has been studied by Eagle (177) who reports that blocking of some of the free amino groups in diphtheria antitoxin or antipneumococcus serum, while inhibiting the flocculating action, did not demonstrably impair the combining affinity of the sera. Treatment with ketene, likewise presumably reacting with amino groups, when not too drastic, alters the antigenic properties without materially weakening the antitoxic (or hemolytic) capacity [Goldie (178)]; a similar result was obtained by Boyd & Tamura (179) with antibacterial sera.

The molecular weight of concanavalin A from jack beans, stated to be a pure hemagglutinin, has been determined as 96,000 in Svedberg's laboratory (180).

Additional papers: Increase of globulin during immunization (181, 46); heat inactivation of various sorts of antibodies (182); increase in production of precipitins by administration of ascorbic acid (183); purification of antitoxins (184, 185).

*Multiple antibodies.*—Goodner & Horsfall (186) have demonstrated by fractional precipitation that horse immune serum to Pneumococcus I contains at least three antibodies which precipitate the specific polysaccharide but differ in their ability to confer passive protection on mice. From the antibodies recovered by dissociating specific precipitates with 10 per cent sodium chloride two fractions were separated, namely, a water-soluble, pseudoglobulin-like antibody, P, with isoelectric point pH 7.6 and low protective capacity, and a water-insoluble, euglobulin-like antibody, E, of high protective power but precipitating less rapidly than P. These antibodies may both be lecithoproteins. (Antibody P reacts with galacturonic acid azoprotein.) Lee, Chow & Wu (187) were able to differentiate fractions in pneumococcus antisera, in particular various anticarbohydrate immune bodies differing in their isoelectric points [*cf.* Felton *et al.* (188)] or in their behavior towards the polysaccharide and its hydrolytic products (*cf.* 166).

As regards the complexity of immune sera with reference to the content of antibodies of divers specificities, two concepts, not mutually exclusive, have been advanced. In the words of Marrack (107):

I. It may be supposed that the antiserum contains two qualitatively different types of antibody molecule, which react with different antigenic groups. The antigenic groups may ( $\alpha$ ) be attached to different molecules, or ( $\beta$ ) to the same molecules. II. It may be supposed that the antibody molecules differ in degree but not in kind, and that some of the molecules react more readily and completely than others with determinant groups differing in composition or arrange-



ment from those of the homologous antigen . . . . Morgan (*cf.* 189) has suggested a hypothesis, intermediate between I and II: that the homologous antigen contains antigenic groups (A,B,C,D,----), of which A is dominant, and that the antiserum contains antibodies (*a*), (*ab*), (*abc*), (*ad*), etc., all reacting with the group (A) . . . .

It has actually been shown (Landsteiner & van der Scheer) that multiple antibodies can be formed to antigens containing a single determinant structure [*cf. Ann. Rev. Biochem.*, 6, 636 (1937)]; the cross-reactions of Pneumococcus-II antiserum with gums are considered by Marrack & Carpenter (107) to be explicable in this way. That on the other hand separate antibodies may be formed to portions of an antigenic molecule has now been definitely shown (190) with compounds possessing two groups known to be serological determinants, *e.g.*, azoproteins containing the residues of phenylarsenic and succinic acids, or of glycine and leucine. In the immune sera, the presence of two separate antibodies was demonstrated by absorption experiments, each antibody being directed towards one of the two groupings. It may be recalled that Haurowitz (191) states that azo-protein immune sera contain antibodies directed respectively towards the hapten, the protein moiety, and apparently both components.

From experiments of Meyer & Pic (192) (absorption of immune bodies to a kaolin-polysaccharide complex, and elution), it is concluded that a single antibody may have affinity for both the lipoidal and the polysaccharide constituents of tubercle bacilli.

#### REACTIONS BETWEEN ANTIGEN AND ANTIBODY

By employing the newer preparations of Pneumococcus I and III specific polysaccharides, which show increased reactivity towards antisera prepared in rabbits and no longer yield zones where both polysaccharide and antibody are simultaneously present, Heidelberger & Kendall (193) found by nitrogen determinations on the precipitates that the same mathematical expressions hold for rabbit sera as for horse precipitins in their reactions with polysaccharides; it is mentioned that more carbohydrate is precipitated by rabbit than by horse antibody. In connection with current ideas on the structure of high molecular polysaccharides, the theory is presented that "the factor determining combining ratios with antibody would not be the molecule as a whole, but the minimum chain length capable of reacting with a single molecule of antibody." This chain weight, *e.g.*, for

Pneumococcus-III polysaccharide, according to tentative calculations, would be 1,800 to combine with one molecule of rabbit antibody and 2,500 for horse antibody. The cross-reactions between Pneumococci III and VIII with respect to agglutinin and anticarbohydrate precipitin in the immune sera were studied quantitatively by Heidelberger *et al.* (194); the same mathematical formulation of antibody reactivity with homologous polysaccharide was applicable to the portion of Pneumococcus-VIII immune body remaining after absorption with Type-III carbohydrate as to the whole VIII antiserum, indicating that both antibody fractions bear like quantitative relations to the homologous polysaccharide. The formulation of the authors for describing precipitin reactions was found to hold also for the reaction between horse-serum albumin and homologous (rabbit) antibody (195), and likewise for the antigen-antibody reactions of the thyroglobulins of several species (196). With this protein, some differences were encountered in quantitative behavior from the systems studied previously, among these being "the extraordinarily great range of combining proportions" (see Table I), and it is pointed out (197) that the high molecular weight and the great deviation from spherical shape of thyroglobulin could indeed afford opportunity for forty to sixty combining groups on the molecule. (The cross-reactions of thyroglobulin from various species are covered by the equation for the homologous reaction.)

From a summary by Heidelberger (197; *cf.* 195) of his formulation of precipitin reactions, the appended table is taken, showing for several systems the composition of the precipitates.

TABLE I

MOLECULAR COMPOSITION OF SPECIFIC PRECIPITATES FROM  
RABBIT ANTISERA

Antigen or Hapten	At extreme antibody excess	At antibody excess end of equivalence zone	At antigen excess end of equivalence zone	In inhibition zone
Cryst. egg albumin (Ea) ..	EaA <sub>5</sub>	EaA <sub>3</sub>	Ea <sub>2</sub> A <sub>5</sub>	EaA <sub>2</sub>
Cryst. serum albumin (Sa) ..	SaA <sub>6</sub>	SaA <sub>4</sub>	SaA <sub>3</sub>	SaA <sub>2</sub>
Thyroglobulin (Tg) .....	TgA <sub>40</sub>	TgA <sub>14</sub>	TgA <sub>10</sub>	TgA <sub>2</sub>
Type-III pneumococcus polysaccharide (S) .....	Sa	S <sub>3</sub> A <sub>2</sub>	S <sub>2</sub> A	S <sub>4</sub> A

A = antibody; S = minimum polysaccharide chain weight reacting. The molecular weight of rabbit-antibody globulin is taken as 150,000.

With azoproteins made from *p*-aminophenylarsenic acid, Haurowitz (198) found these antigens to be precipitated only when the molecule contains at least ten phenylarsenic acid groups, while for the formation of antibodies one arsenic-containing group is sufficient. With azoproteins made from another than the immunizing protein, he found that each determinant group of the antigen combines with one antibody molecule, if not too many (20 to 100) arsenic groups are present in the antigen.

Heidelberger's method of quantitative agglutinin estimation (*cf.* 199) has been extended (200) to the agglutination of "rough" (R) pneumococcal strains of Types I and II with rabbit antisera, the principal antibody of which frequently appeared to be directed toward the non-type-specific somatic carbohydrate "C."

For a study of the composition of specific precipitates in the region of antigen excess, Malkiel & Boyd (201) have utilized two hemocyanins and corresponding rabbit antisera, analyses for copper and nitrogen being made. In this region, unlike the region of antibody excess, the data indicate a linear relation between the amount of antiserum used and the antibody:antigen ratio in the resulting precipitate. (With this high-molecular antigen, at least 36 molecules of antibody seem to be required for the precipitation of one molecule of antigen.) This paper also deals with Hooker & Boyd's theory on the coating of antigen by antibody.

Nitrogen determinations have been employed by Pappenheimer & Robinson (202) for studying the composition of diphtheria toxin-antitoxin floccules. A broad neutral ("equivalence") zone, as in precipitin tests, was found, the results pointing to the combination of toxin and antitoxin in three proportions. The floccules in the equivalence zone contained from 0.5 to 1.4 Lf units of toxin per unit of antitoxin. By removing the soluble toxin-antitoxin complexes by means of precipitins for horse serum, and evaluating their toxicities, Eagle (203) finds that diphtheria toxin and antitoxin "are multivalent with respect to each other." Toxin-antitoxin precipitates produced by means of anti-horse serum could be rendered toxic by excess toxin, then made antitoxic upon exposure to more antitoxin, and so on.

Chow *et al.* (204) report that practically all of the carbohydrate may be recovered by extensive washing of precipitates of Pneumococcus-I polysaccharide and rabbit immune serum, in contrast to the result with horse immune serum precipitates. Of the protein, two-

thirds fails to dissolve despite the removal of carbohydrate. The question of denaturation of antibodies upon combination with antigen receives mention in this and other papers (167, 166, 205).

The argument adduced from the fact that formaldehyde destroys the activity of antibodies cannot by itself be used to prove that union with antigen takes place by virtue of amino groups combining with acid groups in the antigen, since formaldehyde also abolishes the activity of an antibody to a hapten (antipyrine) which, unlike most of those studied hitherto, does not contain any groupings of acid character (149).

The view that the "second stage" of serological aggregation depends solely on specific affinities is opposed by Hooker & Boyd (206) on the grounds that the velocity of flocculation is increased in mixtures of two unrelated flocculating antigen-antibody systems and that agglutinates coalesce even when the cells have been completely coated by antibody; objections are also brought by Eagle (177; *cf.* 166). Experiments upholding the idea of specific forces are presented by Heidelberger & Kabat (199) in the agglutination that occurs between non-sensitized and sensitized pneumococci, when of the same type. The idea is suggested that the long-recognized rôle of electrolytes in agglutination consists in aiding the chemical reaction between antigen and antibody, by minimizing electrostatic effects and providing ions "for the ionized salt complexes in which form antibody probably reacts." Also, experiments by Duncan (207) with velocity determinations similar to those of Hooker & Boyd (206) support the idea of specific forces as primarily active in the second phase, probably helped, in some cases, by non-specific aggregation.

The use of isamine blue has been suggested by Dean (208) as indicator ("like a hemolytic system") for antigen-antibody reactions, the dye entering into the precipitate. For the quantitative determination of hemolytic activity, *cf.* (209).

Experiments on the production *in vitro*, from antigens, of substances supposed to react specifically with the original antigens have been described by Loiseleur (210).

Interesting theoretical discussions on the nature of antigen-antibody reactions, which cannot well be reviewed briefly, are presented by Hooker (211), Morgan (189), and Lettré (212). The theory of the latter author, involving formation of racemates, can hardly explain the reactions of antibodies with haptens not having asymmetric structures.

ALLERGY TO SIMPLE SUBSTANCES<sup>4</sup>

A new instance of simple compounds giving rise to anaphylactic sensitization in guinea pigs, probably due to the formation of conjugates in the body, has been reported by Fierz *et al.* (214)—the sodium salt of 2-carboxy-4-sulfodiaz amino-benzene-4'-arsenic acid decomposing (in acid solution) to the diazonium salt of phenylarsenic acid; shock followed upon injection of an azoprotein from aminophenyl-arsenic acid. The authors were also able (154) to render guinea pigs anaphylactic with an azo dye made from aminosuccinanic acid and resorcinol, shown previously by Landsteiner & van der Scheer to elicit shock in guinea pigs sensitized with the corresponding azoprotein. The effect is ascribed to the formation of azoproteins in the animal body.

Some chlor- and nitro-substituted benzenes, known to sensitize guinea pigs [see *Ann. Rev. Biochem.*, 6, 638 (1937)], were examined by Sulzberger & Baer (215) in human beings. The results were in good agreement with those obtained with animals in that the compounds with labile substituents (1,2,4-chlorodinitrobenzene, and 1,2,4-trinitrobenzene) produced "contact-type eczematous hypersensitivity" in the large majority of the tested individuals. The relationship of this type of sensitivity to frank anaphylaxis in guinea pigs was demonstrated by Landsteiner & Chase (216) in the observation that 1,2,4-chlorodinitrobenzene, a typical incitant of contact dermatitis in man, produces in guinea pigs not only skin hypersensitivity but at the same time, although not regularly, anaphylactic sensitization demonstrable by the Dale technique with protein conjugates. Anaphylactic sensitization was further obtained, and without difficulty, by injections of picryl chloride, and sometimes precipitins for picryl protein were detectable.

In a study by Nitti, Bovet & Depierre (217) of allergic phenomena produced by aromatic amines it was found that unlike *p*-phenylenediamine the ortho and meta isomers are devoid of sensitizing activity, and an analogous relation was observed between hydroquinone on the one hand and catechol and resorcinol on the other. Replacement of a  $\text{NH}_2$  group in *p*-phenylenediamine by  $\text{SO}_2\text{NH}_2$ ,  $\text{CH}_3$  or  $\text{OH}$  abolished the sensitizing capacity; 2-carboxy-1,4-diaminobenzene did not sensitize, while 2-nitro-1,4-diaminobenzene did

<sup>4</sup>For a review on allergic diseases, with comprehensive bibliography, cf. (213).

to a slight degree. Sensitization effects were also obtained with 2-methyl- or 2-methoxy-1,4-diaminobenzene, and 1,2,4-triaminobenzene, with cross-reactions between these compounds and *p*-phenyldiamine. The authors conclude that the allergic effects depend on the reactivity of amino groups. The fact that *p*-sulfamido-chrysoidine sensitizes also to 1,2,4-triaminobenzene, and vice versa, is explained by assuming that the sensitizing power of the former compound depends on its reduction and the formation of triaminobenzene in the animal body (218).

With diazomethane, a substance known occasionally to produce severe allergic symptoms in man, Landsteiner & DiSomma (219) obtained cutaneous sensitization in guinea pigs. They also found it possible, by cutaneous application of mustard oil, to sensitize the skin of hogs and, in one of six cases, that of human beings.

The sodium salt of dichaulmoogroyl- $\beta$ -glycerophosphoric acid has been prepared by Wagner-Jauregg & Arnold (220) and, suggested by the supposed antigenic properties of a phosphatide of tubercle bacilli (containing phthionic acid as fatty acid component), it has been studied as to its antigenicity but the experiments have so far been negative. On treating guinea pigs with another substance containing a fatty acid residue and a sulfonic acid group, oleyl-N-methyltaurine, Fierz *et al.* (221) observed reactions using the Schultz-Dale method; the results, however, were not entirely conclusive. With preparations of tuberculo-phosphatide, hypersensitiveness to tuberculin could not be induced in experiments of Smithburn & Sabin (222) whereas treatment with tuberculo-phosphatide along with protein fractions results in a high degree of sensitivity to tuberculo-protein (223).

Rosenthal (224) has described sensitization of rabbits and guinea pigs to phenolphthalein by treating them with serum containing phenolphthalein in conjugated form. The results, if they can be confirmed, would offer a new approach to the experimental study of drug allergy.

A protein preparation obtained from timothy pollen was found by Harley (225) to contain the skin-reactive substance, a carbohydrate fraction showing less activity. The specific activity is possibly due to a hapten attached to protein (*cf.* 226).

[NOTE: The writers have been informed by Dr. Stuart Mudd that results in the paper quoted as 106, and the second paper under 34, have been found invalid by later work, in particular the statement on a crystalline hemolytic substance obtained from streptococci.]

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# THE BIOCHEMISTRY OF YEAST

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The many references to yeast, found in previous volumes of the *Review*, indicate the increasing importance of the organism as a convenient "bag of protoplasm" with which to investigate the fundamental biological processes both of plants and of animals. The present paper aims to summarize research on the biochemistry of yeast published during the past two years. In the limited space available, no attempt has been made to include all references on the subject; typical contributions have been chosen illustrating present research trends in the field. The topics of enzymes and theories of fermentation have been adequately treated in previous volumes; the present volume also includes several papers dealing with the above subjects. Hence these topics will not be treated in the present discussion.

## THE COMPOSITION OF YEAST

Many researches have indicated that plants contain a nucleic acid (zymonucleic acid) which is different from that found in animal tissues (thymonucleic acid). However, Delaporte & Roukhelman (5) present evidence that both of the above acids are present in yeast: the zymonucleic acid in the nucleic acid extract and metachromatic granules and the thymonucleic acid in the nucleus of the yeast. Woolley & Peterson (76) hydrolyzed yeast with acid and state histidine to constitute about one per cent of the dry yeast; they also isolated small amounts of tyrosine. Myrbäck (42) found tyrosine in autolyzed yeast.

Kraut & Schlottmann (27) analyzed yeast for the biologically important amino acids and compared their ratios with those found in foods of plant and animal origin. They conclude that the proteins of yeast, as a food, occupy a position intermediate between those of plant and animal origin. The high content of cystine and lysine is particularly significant in estimating its value as food for man.

McAnnally & Smedley-MacLean (37) isolated a new carbohydrate from yeast which they consider to be responsible for the immunological properties previously described. The carbohydrate showed sero-



logical properties similar to those of the Type 11 pneumococcus specific polysaccharide but was not identical with it.

Günther & Bonhoeffer (17) and Bonhoeffer & Günther (2) grew yeast in water containing fifty per cent of deuterium oxide with glucose, mannose and levulose as substrates. Various polysaccharide fractions were prepared from the yeast and analyzed for deuterium. Typical results are given below:

PERCENTAGE OF DEUTERIUM

Sugar	Cell Membrane	Yeast Gum	Glycogen
Glucose .....	12.2	11.2	8.3
Mannose .....	15.9	5.4	8.6
Levulose .....	13.1	11.6	4.5

The authors conclude that, if a low deuterium content indicates a more direct synthesis, the cell membrane is most directly synthesized from glucose, yeast gum from mannose and glycogen from levulose. They state that the most direct method for synthesizing a polysaccharide need not start with the hexose representing the building unit since the transformation of one hexose into another is common. The transformations, in so far as they involve enolization, lead to the fixation of two deuterium atoms in each building unit. However, the above process did not wholly explain the total absorption of deuterium.

Reindel, Niederländer & Pfundt (60) made preliminary quantitative studies on the production of sterols by yeast, especially by the so-called "fat" yeasts (*Torula* types). The yield of sterols increased with increasing concentrations of sugar; the highest yield was obtained in a molasses medium. The principal sterol in the yeast was ergosterol, a provitamin D. Wieland, Pasedach & Ballouf (72) isolated cryosterol from yeast and suggested the formula  $C_{30}H_{49}OH$ ; it is a doubly unsaturated alcohol with two double bonds. According to Wieland & Kanoaka (71), zymosterol, obtained from yeast, is a doubly unsaturated alcohol with the formula  $C_{27}H_{45}OH$ ; it is isomeric with ascosterol.

Pavcek, Peterson & Elvehjem (53, 54) studied the effect of various factors upon the growth and vitamin-B<sub>1</sub> content of yeast. Based upon reducing sugar used, the yield of dry yeast was about 30 per cent in the glucose-salts medium, 35 per cent in the molasses-salts medium, and 40 per cent in the grain wort medium. The vitamin-B<sub>1</sub> content of the yeast grown in the above media was, respectively, 3 I.U., 5 I.U. and 10 I.U. per gram of dry yeast. Destruction of the

vitamin in the grain wort medium did not alter either the yield or vitamin potency of the yeast. Omission of aeration of the medium reduced the yield of yeast nine-fold but increased the vitamin potency three-fold. For five strains of yeast tested on the three media, the yield of yeast varied both with the strain and with the medium. The vitamin content was about the same for the several strains on the same medium but varied with the medium employed. Addition of vitamin-B<sub>1</sub> concentrate, crystalline vitamin B<sub>1</sub>, nucleic acid or liver extract to the glucose-salts medium greatly increased the vitamin-B<sub>1</sub> content of the yeast.

Kuhn & Wendt (28) isolated from yeast a compound which possessed the anti-dermatitic properties of vitamin B<sub>6</sub>. The compound has a high molecular weight, is not readily dialyzable and is easily destroyed by heat and alkali. Since its properties differ so widely from those accepted for vitamin B<sub>6</sub>, the authors suggest the name "Adermin-protein" for the compound.

#### FERMENTATION

Guillemet & Leroux (19) found that while yeast alone was not able to ferment inulin, the addition of yeast water permitted a vigorous fermentation; the yeast water alone did not hydrolyze the inulin. The authors conclude that a preliminary hydrolysis of inulin is not necessary to its fermentation by yeast. Haehn, Glaubitz & Gross (20) investigated three species of yeast reported as capable of fermenting dextrins. They found that amyloextrin, the simplest of all dextrins, was not fermented by these species. The authors attribute the confusion in the literature on this subject to difficulties encountered in freeing dextrins from the last traces of sugar.

Genevois (16) found that in every case in which yeast ferments sucrose the sugar is inverted at a rate at least as high as that of the fermentation. At a pH value of 8 yeast will ferment glucose but not sucrose. The optimum pH for the fermentation of sucrose is 4, a value identical with the optimum pH for the action of invertase. The authors present evidence that maltose is fermented directly. Willstätter & Rohdewald (73) are of the opinion that neither dextrose nor maltose is fermented directly but that they are first synthesized to glycogen. Schäffner & Specht (62) found that glycogen, in contrast to dextrose, does not require magnesium ion for its phosphorylation in yeast extracts. Comparative measurements of the disappearance of phosphate and of glycogenolysis showed the former to proceed more

rapidly in the initial stages of fermentation, indicating that phosphorylation precedes glycogenolysis.

Dumazert & Penet (8) studied the fermentation of very dilute solutions of dextrose. They found that the higher the concentration of the dextrose the more nearly does the yield of alcohol approach the theoretical value; for low concentrations of the substrate the relation is very nearly linear. Guillemet & Leroux (18) considered the fermentation balance of dextrose and yeast. There was a residual amount of sugar which was not converted into alcohol, carbon dioxide, glycerol, succinic or acetic acids. The quantities of secondary products of fermentation, from a given amount of sugar, varied inversely with the dry weight of the yeast produced; the quantities of alcohol and carbon dioxide formed were constant.

According to Joslyn & Dunn (23) the volatile acids in a wine-yeast fermentation were found largely in the initial stages of the fermentation when the medium was protected from oxidation. In the presence of oxygen the acids may be formed but they undergo utilization depending upon experimental conditions. The period of formation of the acids corresponds to the stage of decreasing oxidation-reduction potentials.

The work of Hofstetter, Leichter & Nord (22) shows that the heat of reaction of a yeast fermentation varies with the rate of the fermentation, indicating that the fermentation does not follow a fixed single reaction. The heat of reaction for fermentation by yeast maceration juice indicates two different reactions: (a) fermentation of free sugar in the presence of free phosphate (inhibited by phlorhizin) and, (b), fermentation of the remaining substrate in the absence of free phosphate (not inhibited by phlorhizin). Neither reaction shows a thermochemical similarity to that found for fermentation by living yeast. In connection with thermochemical considerations it is interesting to note that Luyet (35, 36) concludes that high pressures and heat flocculate and coagulate the protoplasm of yeast in the same fashion and destroy the permeability of the cell in an analogous manner. The effects of high pressure and heat are additive.

Szilvinyi (68) discusses a procedure for following the oxidation-reduction potential of a suspension of yeast in the presence of varying concentrations of oxygen. The method permits a correlation of the rate of respiration with the rate of reproduction; the higher the respiration the shorter is the generation time. Fromageot & Bost (14) found the  $rH$  values for yeast to vary with the indicator employed.

At  $\text{pH} = 6.4$  the  $\text{rH}$  varies from 4.2 with potassium indigotrisulfonate to 9.7 for phenolindo-2,6-dichlorophenol. The concentration of yeast had little effect at  $\text{pH} = 6.4$  but, in general, the  $\text{rH}$  tended to increase with a reduction in the quantity of the yeast. The  $\text{rH}$  required to reduce acetaldehyde to ethyl alcohol is somewhat less than 7. The authors provisionally accept  $\text{rH} = 4.2$  as representing the maximum reducing power of the yeast.

Kakukawa (24) measured the oxidation-reduction potential of suspensions of living yeast. Under strictly anaërobic conditions both potentiometric and colorimetric methods gave a value of  $\text{Eh} = -170$  to  $-180$  mv. at  $\text{pH} = 5.4$ . (This corresponds to a calculated  $\text{rH}$  of about 5, near that chosen by Fromageot & Bost [14], noted above.) The small amount of oxygen in tank nitrogen stabilized the  $\text{Eh}$  at  $+20$  mv. In the presence of traces of methylene blue or indigo tri- or tetra-sulfonate, the  $\text{Eh}$  fell to the same level with plateaus in the  $\text{Eh}$ -time curves at the normal oxidation-reduction potentials of the indicators; these were completely decolorized at the final  $\text{Eh}$ . Janus green, with a normal potential of  $-140$  mv. was not completely decolorized when the  $\text{Eh}$  became constant at  $-180$  mv. When air was removed by hydrogen the  $\text{Eh}$  fell to the level of the hydrogen electrode but rose to  $-180$  mv. when the hydrogen was replaced by nitrogen.

#### THE NITROGEN METABOLISM OF YEAST

Considerable work has been published dealing with the role of various sources of nitrogen in the metabolism of yeast. According to Edlbacher & Segesser (10) yeast, in a sugar medium, prefers ammonia nitrogen to that furnished by arginine or histidine. In a maceration juice containing yeast, arginine, in the presence of nitrogen, is completely decomposed into urea and ornithine. In the presence of oxygen the arginine is not attacked. In maceration juice, neither histidine nor histamine is acted upon in the presence of oxygen or nitrogen.

Harteljus (21) grew yeast on a mineral-sucrose-ammonium sulfate medium with varying amounts of beer wort, the latter serving as a source of bios. Absorption of nitrogen by the yeast was not much influenced by the concentration of the wort while liberation of nitrogen was lowest in the low concentrations of the wort. Liberation of nitrogen was increased during autolysis; increasing acidity was

probably a factor. Nielsen & Hartelius (49) found that yeast grown in the presence of ammonium sulfate or glycine did not liberate ammonia nitrogen into the medium during growth or autolysis. When grown in excess of these nitrogenous substances, the nitrogen content of the dry yeast was 5 to 6 per cent as compared to 2.5 to 3.0 per cent with an insufficient supply of the source of nitrogen.

Nielsen & Hartelius (46) studied the effect of thirty-four amino acids upon the growth of yeast in a medium containing vitamin B<sub>1</sub> and beer wort. Only  $\beta$ -alanine, asparagine, aspartic acid, lysine and arginine stimulated yeast growth and then only when added in combination; singly the acids were toxic. The  $\beta$ -alanine is regarded as a phytohormone. Under the conditions of the experiments lysine, arginine and glutamic acid could not serve as sources of nitrogen and are regarded as growth substances. Asparagine and aspartic acid probably stimulated growth by virtue of being excellent sources of nitrogen and may more properly be regarded as nutrients.  $\beta$ -Alanine was found (47) to be very toxic toward yeast if asparagine or aspartic acid was not added; that is, asparagine very markedly decreased the toxicity of the  $\beta$ -alanine. The latter compound affected growth only in the presence of asparagine or aspartic acid.  $\beta$ -Alanine could not be replaced by  $\beta$ -alanylglycine or by  $\beta$ -hydroxypropionic acid: the effect on growth depended upon the amino acid itself.  $\beta$ -Alanine and aspartic acid were prepared by the hydrolysis of  $\beta$ -alanylglycine and glycylaspartic acid, respectively; the acids so produced showed a growth effect. Both  $\beta$ -alanylglycine and glycylaspartic acid themselves were without effect, hence the action of the amino acids cannot be explained on the basis of a preliminary combination to form peptides.

Nielsen (43) determined the percentages of the following amino acids assimilated by yeast: *dl*- $\alpha$ -aminobutyric, *dl*- $\alpha$ -aminocaproic, *dl*- $\alpha$ -aminocaprylic, N-methylglycine, N-dimethylglycine, *dl*- $\alpha$ -aminoisobutyric, and *dl*- $\epsilon$ -aminocaproic. He concluded that the group  $\text{HC}(\text{NH}_2) =$  is necessary for the assimilation of amino acids by yeast. Thorne (70) investigated the action of yeast on six amino acids. He confirmed the theory of Ehrlich that yeast assimilates the amino nitrogen by splitting off the amine group from the acid. This action, together with the splitting off of carbon dioxide from the carboxyl group, gave the corresponding alcohols which were isolated and identified.

## FACTORS INFLUENCING THE GROWTH OF YEAST

The chemicals to be discussed affect the reproduction of yeast but do not serve directly as nutrients. Those which increase the rate of reproduction have been variously called growth-promoting substances, cell-proliferation stimulants, growth stimulants and food-accessory materials. The terms growth, reproduction, multiplication and proliferation have been used interchangeably with reference to microorganisms, especially yeast. Strictly speaking, the term growth refers to the increase in weight, or volume, of a cell or community of cells. The terms reproduction, multiplication and proliferation refer to that process by which one living unit becomes two individuals. However, with such an organism as yeast the curve which expresses the number of organisms as a function of time (reproduction), in a given medium, parallels the time curve for total crop (growth); that is, for comparative purposes the weight of cells, growth, is proportional to the number of cells present in a given medium. Since rates are usually to be compared, the use of the term growth has some justification though applied to changes in the number of cells. Moreover, the term growth is so generally employed as synonymous with reproduction that a complete revision of usage is at present not justified. However, for those not acquainted with this connotation the above explanation, or apology, is deemed advisable.

There has been some discussion as to whether the cell division of yeast is mitotic or amitotic. Colchicine is known to delay mitotic division at the metaphase and has been employed in studies of cell division of both plant and animal cells. A proper concentration of the drug should delay any mitotic division of yeast, change the rate of growth and permit the observation of division figures. However, Richards (61) found that colchicine actually stimulates the growth of yeast thus failing to show that mitosis takes place. The stimulation was not the kind shown by a bios but was due to the drug serving as a nutrient and as a buffer in lessening the adverse effect of the increasingly unfavorable medium on the growth of yeast populations.

Winge & Laustsen (74, 75) studied the problem of haplophase and diplophase in the *Saccharomycetes* and showed that genetic segregation takes place in bakers' yeast when ascospores are isolated with a micromanipulator. They record the first successful experiment in producing a species hybrid within the genus *Saccharomyces*. Spores of *Saccharomyces ellipsoideus* and of *S. validus* were placed, in pairs, within droplets of the culture medium. A hybrid-zygote was pro-

duced and from the zygote a diploid yeast hybrid was grown. The appearance of the hybrid was intermediate between that of the parental types; spore formation of the hybrid was good but the germination power of the isolated spores was very poor. The hybrid showed segregation in the progeny from single spores, but the segregated types produced very slow-growing giant colonies. The hybrid itself was very vigorous in growth, probably due to heterosis. Rates of growth and of fermentation as well as dry weight production were higher than with the parental species. This research is important in showing the possibility of scientific work in the cross-breeding of yeasts to produce hybrids.

Miller (41) has reviewed the work in his laboratories dealing with various bios fractions. Detailed methods are given for the purification of Bios IIB. Bios V was found to be identical with vitamin B<sub>1</sub>. With *Saccharomyces valbyensis* neither crude Bios IIA nor crude Bios IIB could be replaced by purified preparations which gave good crops with *S. cerevisiae*; each of the crude preparations contained a stimulant effective with the former species but not with the latter. The provisional name, Bios VII, is proposed for the above factor accompanying Bios IIB. Lactoflavin (riboflavin), a member of the vitamin-B<sub>1</sub> complex, could not replace crude Bios IIA, IIB or V; choline hydrochloride likewise proved inactive. Carnosine nitrate could not replace Bios IIA or IIB. Histidine hydrochloride did not affect the crop of *S. cerevisiae*.

Eagles, Okulitch & Kadzielawa (9) studied three distinct growth accelerants, prepared from tomatoes, upon two species of lactic acid bacteria. One of the activators was Bios IIA and the other two were components of Bios IIA. On the basis of their physical and chemical properties they suggest that the stimulants required for the bacteria are identical with certain heat resistant accessory food factors of the vitamin-B complex.

Laird & West (29) fractionated bios by the procedure of Miller and coworkers and found some of the fractions capable of replacing yeast extract in culturing strains of *Rhizobium trifolii*. The effect was measured by acid production. Bios I (*i*-inositol) was inactive while Bios IIB, V and IIA showed definite production of acidity, the potency increasing in the order named. These effects could not be brought about in the synthetic media by addition of crystalline vitamin B<sub>1</sub>, nicotinic acid, uracil, choline,  $\beta$ -alanine, carnosine,  $\beta$ -indole acetic acid,  $\beta$ -indole butyric acid, glutathione, cysteine or vitamin C.

Kögl & Fries (26) studied the effect of biotin, aneurin and *i*-inositol upon the growth of various fungi on synthetic media. The phycomycetes tested required aneurin, and growth was not further increased by biotin and inositol alone or in combination. The ascomycetes tested required biotin and inositol for appreciable growth;  $\beta$ -alanine was without effect. That is, the organisms differed markedly in their reactions toward the above reagents. Pairs of fungi with complementary requirements can be grown without the addition of the other factors, thus furnishing one possible explanation of symbiosis.

Schultz, Atkin & Frey (64, 65) found that vitamin B<sub>1</sub> (Merck's natural crystalline) in a concentration as low as  $10^{-6}$  grams per 100 cc. of medium increased the rate of production of carbon dioxide in a yeast fermentation. Synthetic vitamin B<sub>1</sub> (Merck's Betabion) gave identical results. They claim the assay of vitamin B<sub>1</sub>, by the above fermentation method, to be rapid and reliable for control purposes and to give good correlation with results of rat-feeding experiments. Thiamine and 2-methyl-5-ethoxymethyl-6-aminopyrimidine likewise increased the rate of carbon dioxide production in the yeast fermentation.

In studies on the biocatalysts of yeast Medvedev & Wysotskaja (40) found that the addition of yeast autolysate to washed yeast, in the presence of glucose, increased the rate of production of carbon dioxide. The bios or Z-factor involved is directly proportional to the amount of yeast employed. In addition to other factors claimed to be necessary for the growth of yeast, Devloo (6) found a sterol, "biosterol," present in crude biotin and ordinary sugar. Cholesterol itself was inactive but usually contained the "biosterol." Ergosterol, sitosterol and vitamin D<sub>2</sub> were as active as the "biosterol" but were different in properties.

According to Loofbourow, Schmieder, Stimson & Dwyer (34) the spectrum of a bios preparation from yeast resembles that of liver extract, both having a marked maximum at 2600 Å, which is characteristic for nucleic acid and pyrimidine constituents. The spectrum of the bios preparation from malt combings lacked any marked extinction at 2600 Å but, instead, showed peaks at 2800 and 3000 Å, which may be accounted for in part by the presence of amino acids. The spectrum of bios from sucrose showed only "end" absorption. They conclude different materials to be present in at least three of the preparations.



Fulmer, Underkofler & Lesh (15) found that, with the strain of yeast employed, the presence of magnesium sulfate markedly increased the growth in the presence of Bios II. Magnesium chloride or nitrate did not show this effect while ammonium sulfate gave some increase in the multiplication. Combinations of magnesium chloride or nitrate with ammonium sulfate gave about the same increase in growth as did the magnesium sulfate. The studies were extended (31) to include the effect of Bios II, Bios I (*i*-inositol) and magnesium sulfate, alone or in combination, upon thirteen strains of *Saccharomyces cerevisiae*. The various strains showed marked differences in response and were grouped into three main groups. *Group I*:—The addition of magnesium sulfate with Bios II did not give increased growth. *Group II*:—The addition of Bios I with Bios II did not give increased growth. *Group III*:—Growth was increased under the conditions given for Groups I and II. The above groupings show that discrepancies in the literature on bios may be largely due to differences in the strains of yeast employed and in the composition of the medium. For example, if a strain of Group I were grown in a medium containing magnesium sulfate, Bios II would give increased counts in the presence of Bios I, that is, the complementary effect of Bios I and Bios II, described by Miller and coworkers, would be demonstrated. If, however, a yeast of Group II or Group III were employed under the same conditions, Bios II would be potent in the absence of Bios I and the complementary effect would not be evident.

Schultz, Atkin & Frey (66) divide yeasts into two classes on the basis of their response to various growth stimulants. Type A cultures (*Luft* II and *Rasse* XII) are stimulated by thiamin; Type B cultures (*Saccharomyces cerevisiae* Toronto Spc. 152) are inhibited by this reagent. The above is true also in the presence of Bios I (*i*-inositol), Bios IIA ( $\beta$ -alanine) and Bios IIB. 4-Methyl-5- $\beta$ -hydroxyethylthiazole and 2-methyl-5-ethoxymethyl-6-aminopyrimidine stimulate Type A yeast while Type B is not affected by the first compound and is inhibited by the second; the combination of the above reagents inhibits Type B to the same extent as does thiamin.

Schopfer (63) found that a species of *Rhodotorula* grew readily in a medium free from auxin materials. Its growth was not stimulated by panthothenic acid with or without the addition of inositol while *Saccharomyces cerevisiae* responded in the expected manner. The *Rhodotorula* species contained Bios III, replaceable by aneurin, as a cell constituent; that is, the organism was capable of synthesizing

it. *Rhodotorula rubra* and *Rhodotorula flava* were stimulated by thiazole and pyrimidine; the effect was not additive.

Norris & Ruddy (52) and Norris & Kreke (51) claim that, contrary to the observations of some earlier workers in the field, bios has the power to stimulate the endogenous respiration of yeast; excess sugar obscures this action. They conclude that there are three separate factors responsible for the stimulation of respiration, growth and fermentation. These factors are all dialyzable according to Norris & Hart (50).

Nielsen & Hartelius (48) state that the growth-promoting factors which influence cell production or the production of cell substance in the lower plants are different from those which influence cell elongation in the higher plants. The authors place materials affecting cell elongation, for example auxin and  $\beta$ -indolyl acetic acid, in Group A while other growth-promoting materials which differ from these in chemical composition and physiological action are placed in Group B. Members of the latter are further divided into two sub-groups:—B<sub>1</sub>, those which are easily oxidized and act upon yeast; B<sub>2</sub>, those which are very resistant toward oxidation and act upon *Aspergillus niger* and other molds. Such large quantities of B<sub>1</sub> materials are synthesized by the molds that their addition to the medium is unnecessary; large quantities of Group A materials, for example,  $\beta$ -indolyl acetic acid, are also produced. Nielsen & Fang (44, 45) presented further evidence that Group B contains materials one of which affects the growth of yeasts and not molds while another affects molds and not yeasts. They shook six species of yeast with beer wort and found the yeast to quantitatively remove group B<sub>1</sub> materials, while the content of group B<sub>2</sub> materials was unchanged.

Sperti, Loofbourow & Dwyer (67) found that yeast cells injured by ultraviolet light produce materials which act as stimulants to the growth of yeast. The phenomenon is not associated with substances normally found in the cells, but the factor involved is apparently formed by the living cells as a result of the injury. The results are discussed with reference to their implications in wound healing, presenting a possible mechanism permitting the cell community to respond to sudden changes in conditions in such a manner as to preserve community life. They provisionally class such substances as "wound hormones." The spectrographic studies of Loofbourow, Cook & Stimson (32) on the proliferation-promoting substances produced by yeast cells injured by ultraviolet light, indicate the presence of phos-

phorus, pentoses, guanine and adenine. Uracil, cytosine, pyridine, and vitamins B<sub>1</sub> and B<sub>2</sub> are not indicated as being present; yeast nucleic acid and adenylic acid seem to be ruled out by the Thomas (69) test.

Farndon, Carroll & Ruddy (12) found the respiration of yeast cells to be decidedly stimulated by irradiation with ultraviolet light or x-rays. However, the fact that this stimulation could be demonstrated not only by irradiated suspensions, but also in non-irradiated suspensions to which irradiated or heat-treated cells were added, indicates that the increase in respiration is not the result of direct action by the radiation. It is caused rather by materials produced from cells destroyed by heat or by irradiation. Farndon & Ruddy (13) tested both centrifuged and filtered fluids from irradiated yeast for their power to stimulate the respiration of non-irradiated yeast. The effect of the cell-free fluid was pronounced while that of the washed cells was negligible. Ringer solution, or more specifically the glucose in the solution, when irradiated with ultraviolet light had some power to stimulate respiration. Cook, Hart & Joly (3) found the above factor to stimulate the endogenous and exogenous respiration of yeast to the same extent.

Leonian & Lilly (30) found heteroauxin to act as an inhibitor, rather than as a growth-promoting substance, to various fungi. According to Loofbourow & Dwyer (33) the addition of heteroauxin inhibits the growth of yeast but also causes the injured cells to produce a "wound hormone" which stimulates the growth of untreated yeast. They conclude that the action of heteroauxin on yeast is consistent with the mode of action suggested by Leonian & Lilly (30) who concluded that:

It may be possible that acting like a powerful stimulant or irritant, it (heteroauxin) induces the formation, the transportation to, and the concentration in the invaded regions of large quantities of the growth substances of the plant.

Kimball (25) presents evidence showing that when old yeast cells, growing on a favorable medium, are placed in the magnetic field of a small horseshoe magnet the rate of bud formation is retarded by about thirty per cent. The cells are sensitive to this action only during the last half of the lag phase. The phenomenon is apparent only in a heterogeneous field, a gradient being necessary.

According to Malkov & Mesoustuk (38) the rate of multiplication of yeast is increased by a preliminary treatment with a solution of phosphate before placing in a nutrient medium. The authors ascribe the effect to the formation of non-ionized complexes with the

intra- and extra-cellular iron, leading to lower oxidative changes during the early stages of growth. These complexes slowly break down and liberate highly active iron, as a result of which the metabolic activity of the cells is maintained at a high level for a long period of time.

Arloing, Morel, Josseraud & Perret (1) state that sodium ascorbate has no effect upon carbon dioxide production by yeast; sodium dehydroascorbate had a weak inhibiting action. Ferrous and ferric chlorides and ferrous complexes of ascorbic acid were slightly inhibitory while the ferric complex of dehydroascorbic acid was without effect. Mameli & Baratto (39) found that small amounts of ascorbic acid,  $\beta$ -carotene, acamnine, adenine, phytin or inosine accelerated the fermentation rate by yeast for the first two hours. In every case the acceleration was increased when the compound was first exposed to ultraviolet light.

Cook, Hart & Joly (4) found that 1,2,5,6-dibenzanthracene increases the proliferation of yeast; the maximum increase was 50 percent at a concentration of  $9 \times 10^{-4}$  molar. Similar concentrations of anthracene were without effect. Dodge & Dodge (7) studied the effect of methylcholanthrene upon the morphology and growth of six yeasts. With *Saccharomyces ellipsoideus* the total dry weight and the rate of fermentation were increased about one-third in a peptone-glucose medium saturated with the methylcholanthrene. After two months of culturing, giant cells and increased differentiation were found in the colony.

According to Enders & Wieninger (11) the following alkaloids inhibit growth and fermentation by yeast in the order named:—quinine, caffeine, cinchonine, and pilocarpine. This is not the same order as that for the relative toxicities of the alkaloids toward the animal organism. In general, fermentation is inhibited by a lower concentration than that required to inhibit the power to multiply.

Pourbaix (55, 56, 57, 58, 59) studied the effect upon the growth of yeast of the carcinogenic compound styryl 430 (2-*p*-acetylaminobenzoylaminoquinoline methoacetate). At a concentration of 0.01 per cent the compound materially decreased the oxygen consumption and production of carbon dioxide for a forty-eight hour period, after which the yeast gradually recovered. Addition of phosphate tended to overcome the effect by precipitating the compound. Yeast fermentation, in the presence of the styryl, was much less sensitive to sodium fluoride than in its absence. If the synthesis of adenosine triphos-

phoric acid is inhibited by the compound, the addition of adenylic acid and magnesium chloride should make the yeast more sensitive to the fluoride; such proved to be the case. The filtrate from boiled yeast contained a substance which counteracted the effect of the styryl. The "antistyryl" was found to be insoluble in acetone and ethyl alcohol; it is not nicotinic acid, hexosediphosphate, vitamin B<sub>1</sub> or vitamin B<sub>2</sub>.

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